

IN VITRO SEED GERMINATION AND SEEDLING DEVELOPMENT OF *Calopogon tuberosus* AND *Sacoila lanceolata* var. *lanceolata*: TWO FLORIDA NATIVE TERRESTRIAL ORCHIDS

By

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by

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This document is dedicated to all native orchid enthusiasts.

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TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT	x
CHAPTER	
1 LITERATURE REVIEW	1
Methods of Orchid Propagation.....	2
Environmental Factors Effecting Seed Culture of Temperate Terrestrial Orchids	6
Plants of Study	10
<i>Calopogon tuberosus</i> var. <i>tuberosus</i>	10
<i>Sacoila lanceolata</i> var. <i>lanceolata</i>	11
Seed Culture of <i>Calopogon tuberosus</i>	19
2 SEED CULTURE AND SEEDLING DEVELOPMENT OF <i>Calopogon tuberosus</i>	23
Introduction.....	23
Materials and Methods	25
Media Preparation	26
Light Treatments	26
Seedling Development.....	27
Acclimatization Study	30
Statistical Analysis	31
Results.....	32
Effect of Light Treatment and Culture Media	32
Qualitative Assessment of Media and Light Interactions on Seed Germination and Seedling Development	41
Seedling Development.....	42
Observations on Cold-Storage.....	47
Acclimatization.....	48
Discussion.....	49
Media Comparison	49
Light Treatment Effects.....	66

Cold-Storage.....	67
Acclimatization.....	68
Corm Formation.....	69
Conclusions and Recommendations.....	70
3 SEED CULTURE AND SEEDLING DEVELOPMENT OF <i>Sacoila lanceolata</i> var. <i>lanceolata</i>	72
Introduction.....	72
Materials and Methods	74
Media Preparation	74
Light Treatments	75
Statistical Analysis	76
Results.....	76
Discussion.....	77
Conclusions and Future Implications	83
LIST OF REFERENCES.....	84
BIOGRAPHICAL SKETCH	91

LIST OF TABLES

<u>Table</u>	<u>page</u>
1-1 Composition of media commonly used for <i>in vitro</i> seed culture of orchids	9
2-1 Formulations of orchid seed sowing medium (P723), Malmgren modified terrestrial orchid medium (MM), and Modified Knudson's C (KC) medium as prepared by <i>Phytotechnology</i> Laboratories.....	28
2-2 Comparative mineral salt content of orchid seed sowing medium (P723), Malmgren modified terrestrial orchid medium (MM), and Knudson C (KC) as prepared by <i>Phytotechnology</i> Laboratories.....	29
2-3 Six stages of orchid seed development	30

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
1-1 Distribution of <i>Calopogon tuberosus</i> var. <i>tuberosus</i>	13
1-2 Growth habit of <i>Calopogon tuberosus</i>	14
1-3 Various pink flower forms of <i>Calopogon tuberosus</i>	15
1-4 White flower color of <i>Calopogon tuberosus</i>	16
1-5 Distribution of <i>Sacoila lanceolata</i> var. <i>lanceolata</i>	17
1-6 Growth habit of <i>Sacoila lanceolata</i> var. <i>lanceolata</i>	18
2-1 Effect of culture media and light treatment on percent seed germination of <i>Calopogon tuberosus</i> after 8 weeks culture	35
2-2 Effect of culture media on maximum germination rates of <i>Calopogon tuberosus</i> ..	36
2-3 Effect of light treatment and culture media on <i>in vitro</i> development of <i>Calopogon tuberosus</i> protocorms and seedlings.....	37
2-4 Effect of culture media on weekly protocorm and seedling development of <i>Calopogon tuberosus</i>	38
2-5 Effects of culture media and dark pretreatment on weekly seed germination of <i>Calopogon tuberosus</i>	39
2-6 Effect of media and dark pretreatment on seedling development after 8 weeks culture.....	40
2-7 Stages of <i>in vitro</i> seed germination and seedling development of <i>Calopogon</i> <i>tuberosus</i>	43
2-8 Comparative effects of media and light on protocorm and seedling development of <i>Calopogon tuberosus</i> after 8 weeks culture.....	44
2-9 Seed germination and seedling development of <i>Calopogon tuberosus</i> after 8 weeks culture.....	45

2-10	Comparative effect of media and light treatment on seed development of <i>Calopogon tuberosus</i> after 12 weeks culture	46
2-11	Comparative effect of media and light treatment on leaf and root length of <i>Calopogon tuberosus</i> seedlings after 24 weeks culture	50
2-12	Comparative effect of culture media and light treatment on leaf and root number of <i>Calopogon tuberosus</i> seedlings after 24 weeks culture	51
2-13	Comparative effect of culture media and light treatment on fresh weight of <i>Calopogon tuberosus</i> seedlings after 24 weeks culture	52
2-14	Comparative effect of culture media and light treatment on dry weight of <i>Calopogon tuberosus</i> seedlings after 24 weeks culture	53
2-15	Comparative seedling development of <i>Calopogon tuberosus</i> after 24 weeks culture	54
2-16	Seedling development of <i>Calopogon tuberosus</i> in Phytotech Culture Box after 16 weeks culture	55
2-17	Corm development after 7 months <i>in vitro</i> culture of <i>Calopogon tuberosus</i> seedlings	56
2-18	Effects of cold-storage on seedlings of <i>Calopogon tuberosus</i>	57
2-19	Potting media effects on leaf and root production of <i>Calopogon tuberosus</i> seedlings acclimatized to greenhouse conditions for 12 weeks	58
2-20	Potting media effects on leaf and root length of <i>Calopogon tuberosus</i> seedlings acclimatized to greenhouse conditions for 12 weeks	59
2-21	Potting media effects on fresh and dry of <i>Calopogon tuberosus</i> seedlings acclimatized to greenhouse conditions for 12 weeks	60
2-22	Potting media effects on corm diameter of <i>Calopogon tuberosus</i> seedlings acclimatized to greenhouse conditions for 12 weeks	61
2-23	Twelve-week greenhouse acclimatization of <i>Calopogon tuberosus</i> seedlings	62
3-1	Effects of media type and light treatment on seed germination and seedling development of <i>Sacoila lanceolata</i> var. <i>lanceolata</i>	78
3-2	Effects of media type and light treatment on weekly seed germination of <i>Sacoila lanceolata</i> var. <i>lanceolata</i>	79
3-3	Seed germination and seedling development of <i>Sacoila lanceolata</i> var. <i>lanceolata</i>	80

Abstract of Thesis Presented to the Graduate School
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IN VITRO SEED GERMINATION AND SEEDLING DEVELOPMENT OF *Calopogon*
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TERRESTRIAL ORCHIDS

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The increased popularity of orchids has lead to a major increase in production and sales. With increased popularity a potential need exists for the introduction of new commercially valuable orchids. Recently commercial production and cultivation of native terrestrial orchids have slowly increased. A major obstacle to native orchid production is the difficulty in seed germination. Many cold-hardy terrestrial orchids are difficult to propagate from seed, while only a few are easy to germinate.

Two native orchids with horticultural potential are *Calopogon tuberosus* and *Sacoila lanceolata*. Several small-specialized nurseries offer *Calopogon tuberosus*, but *S. lanceolata* is not commercially available. Native orchids are produced through seed germination, but seedling development can be a long process and flowering plants are often produced only after 3-5 years of growth. Seed germination research has been common for *C. tuberosus*, but no studies exist for *S. lanceolata*. This study was

undertaken to improve existing seed germination protocols for *C. tuberosus*, as well as create a protocol for germination *S. lanceolata* seeds.

Seeds of each species were cultured on three culture media in combination with 5 light conditions. Culture media included Knudson C (KC), Malmgren modified terrestrial orchid medium (MM), and *Phytotechnology* orchid seed sowing medium (P723). Light treatments included 8 weeks continual darkness, an 8-week 16-hr photoperiod, 2 weeks dark followed by 6 weeks light, 4 weeks dark followed by 4 weeks light, and 6 weeks dark followed by 2 weeks light.

Seed germination of *S. lanceolata* was extremely low, with only one seedling developing within 5 months. Seeds of *C. tuberosus* germinated in all light treatments; however, seed germination was highest on KC in 8 weeks dark. Seedlings developed rapidly on P723 in the 8-week 16-hr photoperiod. This suggests that while KC and darkness promoted seed germination, P723 and light were more beneficial for seedling development. Seedlings of *C. tuberosus* also were easily cultivated under greenhouse conditions. This study presents the first step in developing protocols to produce native orchids in shorter time periods.

CHAPTER 1 LITERATURE REVIEW

The Orchidaceae, comprising approximately 10% of angiosperms, is the largest family of plants with 20,000 to 30,000 species with a holarctic distribution (Cronquist, 1981; Dressler, 1981). Members of the Orchidaceae are among the rarest plants such as *Cypripedium calceolus* in Europe (Ramsay and Stewart, 1998) as well as common species as *Calopogon tuberosus* in Eastern North American. Approximately 70% of orchid species have an epiphytic growth habit (Royal Botanic Gardens, Kew, 2003). As an adaptation to wind dispersal, orchid seeds are minute varying in size from 150 to 6,000 μm (Molvray and Kores, 1995). The seeds contain a small embryo and lack enzymes to metabolize polysaccharides, but utilize lipids as a major nutrient source. The embryo also lacks enzymes to convert lipids to soluble sugars (Manning and van Staden, 1987). Due to the lack of enzymes, orchid seeds require a symbiotic relationship with a mycorrhizal fungus in order to germinate under natural conditions (Rasmussen et al., 1990). This fungal association provides the seed with carbohydrates, nutrients, minerals, and water (Rasmussen, 1992).

Evident by the recent increases in wholesale prices, orchids are now the second most popular potted floriculture crop (United States Department of Agriculture [USDA], 2004). Wholesale prices in 2000 were estimated at \$100 million increasing to approximately \$128 million in 2004. This is a substantial increase from wholesale prices of \$46 million in 1996 (USDA, 2004). On the retail level orchid sales are estimated to be worth up to \$250 million (Nash, 2003). Recent articles have mentioned the tropical

epiphytic genus *Phalaenopsis* and its hybrids as the most popular orchid, making up 75 to 90 percent of all orchid sales in the United States (Griesbach, 2002; Nash, 2003). Other common orchid genera and hybrids including *Dendrobium*, *Cattleya*, *Cymbidium*, *Epidendrum*, *Oncidium*, *Phaius*, *Spathoglottis*, and *Vanda* are readily available at discount department and home-improvement stores. The production of potted orchids has increased due to growth in popularity, advances in propagation techniques, perception that potted orchids can be profitable for commercial growers, segmentation of the market, and reduction in price of individual potted plants (Britt, 2000; USDA, 2004).

Methods of Orchid Propagation

In nature orchid seeds germinate only following infection with a mycorrhizal fungus (Rasmussen, 1992.). The fungus provides the embryo with organic material, water, and mineral nutrients (Smith, 1966). Although seeds lack large amounts of carbohydrate reserves, starch is accumulated upon hydrolyzation of lipids, proteins, and glycoproteins (Manning and van Staden, 1987). Upon penetration by the fungus into the seed, fungal hyphae are digested providing a source of exogenous carbohydrates to the developing embryo. Since germination begins at imbibition, the fungi may serve as a water supply (Yoder et al., 2000).

In the late 1800s researchers began to investigate the subject of germinating orchid seeds. Seed was often placed on organic substances such as sphagnum moss, bark, or leaf mold in anticipation of seed germination, but this often proved unsuccessful (Arditti, 1967a). Seed was also frequently placed on the compost in the pot of the seed capsule parent (T. Sheehan, pers. comm.). Several early researchers such as Bernard and Burgeff explored the symbiotic relationship between orchids and mycorrhizal fungus, and concluded that under *in vitro* conditions orchid seeds germinated only in the presence of a

symbiotic fungus. Although it was widely accepted that orchid seeds could only be germinated with the proper fungus, germination rates were often low and seedling death was common (Knudson, 1922). Bernard, however, was successful in germinating seeds of *Cattleya* and *Laelia* in the absence of a fungus by using salep, a powder obtained from tubers of *Ophrys*, a terrestrial orchid genus (Arditti, 1967a; Knudson, 1922).

Based on initial experiments by Bernard and Burgeff, Lewis Knudson further examined the role of fungi in orchid seed germination. Knudson (1922) stated that germination might not be induced by the “fungus within the embryo, but by products produced externally upon digestion of the fungus.” Using a nutrient solution based on Pfeffer’s solution, Knudson (1924) successfully germinated seeds of several epiphytic orchid genera in the absence of a mycorrhizal fungus. From these initial experiments, Knudson developed Solution B (Table 1-1), and proved that orchid seed could germinate *in vitro* in the absence of a symbiotic fungus. Knudson (1946) modified Solution B in order to germinate seeds of more difficult genera and species. Knudson added manganese and replaced ferric phosphate with ferrous sulfate. The medium, referred to as Knudson C (Table 1-1), is widely used for both plant tissue culture and *in vitro* seed germination.

With the advent of asymbiotic seed germination came an interest in germination of native terrestrial orchids. Since many native orchids are rare, threatened, or endangered, seed germination has been applied to species conservation. Through *in vitro* seed culture a diverse gene pool can be maintained and preserved (Stenberg and Kane, 1998). Although conservation is still a primary goal of native orchid seed culture, a recent purpose is to provide plants to the consumer to alleviate collection pressure

(Anderson, 1990; Brumback, 1990; and Lighty, 1990). Hardy terrestrial orchids are adapted to variable habitats (Stoutamire, 1983) making the plants suitable for a wide range of garden conditions. Lack of knowledge and interest by the consumer and industry, difficulties in propagation methods, and a long maturation process has limited the market for native orchids. Recent advances in the application of seed culture have lead to an expanding market; however, small hobby growers make up the majority of the market, and availability of numerous native orchid genera is relatively small.

Two early researchers of native orchids were Curtis and Carlson. Curtis was one of the first to develop a culture medium specific for the germination of native terrestrial orchids (Curtis, 1936) (Table 1-1). Curtis germinated 31 species native to Wisconsin on this medium, and eight species developed into green seedlings within two years. Several years later, Curtis developed a medium specific for the genus *Cypripedium* (Curtis, 1943).

While Curtis emphasized the germination of orchid seed, Carlson focused on the anatomy of developing seeds and seedlings. Two early studies focused on the germination and seedling development of *Calopogon pulchellus* (*Calopogon tuberosus*) (Carlson, 1936, 1943). Carlson (1936) found that *C. pulchellus* germinated on Knudson B, and also provided the first micrographs of germinating seeds and developing seedlings. Although picture quality was poor, this provided the only published detailed outline of *Calopogon* seed germination. Carlson (1943) presented a more detailed study of the anatomy of *C. pulchellus*. The development of seedlings was divided into the first two years of growth followed by development of mature plants, root structures, leaves,

and vasculature. These studies were valuable since they presented detailed development of native orchid seed.

Germination difficulties are well documented with native terrestrial orchids. Stoutamire was one of the first to address these difficulties. Stoutamire (1964) concluded that dormancy mechanisms exist in mature seeds that lead to decreased germination; however, the exact function of this dormancy is not well understood. Stoutamire (1964) found that twenty species did not germinate on Knudson C, and speculated that this could be due to 1) loss of germination capacity in stored seed 2) embryo sensitivity to surface sterilization techniques 3) lack of required growth regulators for germination 4) lack of a required cold treatment before inoculation. Most seeds germinated poorly, but seeds that germinated quickly included *Calopogon*, *Platanthera*, *Pogonia*, and *Spiranthes* species. Stoutamire (1974) observed that native terrestrial species may flower as quickly as two years or take as long as eight years after seed germination, with most species requiring four to five years.

Due to difficulties with *in vitro* seed culture of terrestrial orchids, many researchers formulated media specifically for terrestrial orchids. Several media that have proven valuable include Fast medium (Fast, 1976) developed for *Cypripedium*, BM developed for Western European orchids (Van Waes and Debergh, 1986b), and Curtis medium (Curtis, 1936). While researchers in academia developed these media, recently hobbyists have also contributed to media formulation. Media developed by hobbyists are often overlooked and not used in published research. Malmgren, a hobbyist, developed a medium for germinating European terrestrial orchids (Malmgren, 1996) (Table 1-1). Malmgren suggested that the addition of a complex undefined organic substance, such as

pineapple juice, contributes unknown sugars, vitamins, minerals, and hormones, which are beneficial for germination. The medium did not contain an inorganic nitrogen source such as ammonium nitrate or ammonia sulfate, but the amino acid solution, Vamin. The amino acids might have low toxicity, which may be beneficial for species that require long incubation period, and Malmgren reported high seedling survivorship on this medium.

Culture media not specifically developed for orchid seed germination have also been widely used. Murashige and Skoog medium (1962), commonly referred to as MS medium (Table 1-1), developed for proliferation of tobacco callus, is also used for seed germination. Since MS medium has a relative high macro and micronutrient content, it is often used in half or quarter strength. *Phytotechnology* Laboratories (Shawnee Mission, KS) has developed several orchid seed culture media based on MS medium in both half and quarter strength including P723 and P748.

Environmental Factors Effecting Seed Culture of Temperate Terrestrial Orchids

Since terrestrial orchids are adapted to variable habitats, optimal *in vitro* culture conditions suitable for one species may differ for other species (Van Waes and Debergh, 1986b). One such culture condition is photoperiod. Debate remains whether complete darkness or some type of illumination is optimal for seed germination of temperate terrestrial orchids. Although the role of photoperiod may be important, this effect has not been explored thoroughly in asymbiotic germination or used with proper statistical analysis.

Oliva and Arditti (1984) reported that photoperiod did not have a significant effect on germination of several species of *Aplectrum*, *Spiranthes*, and *Cypripedium*. Arditti et al. (1981) reported that illumination negatively affected the germination of *Calypso*

bulbosa and *Epipactis gigantea*; however, no difference was found between illumination and complete darkness in seed germination of *Goodyera*, *Piperia*, and *Platanthera*. In both studies the exact length of photoperiod was not included.

Stoutamire (1974) reported that a 12-hour light/12-hour dark photoperiod negatively affected germination of several temperate terrestrial species. Of 18 species examined, one species developed into a 2 mm protocorm within 10 months. Under complete darkness, embryos developed into seedlings with 1 cm leaves in 10 months. Stoutamire hypothesized that light-inhibition may be part of a protective mechanism. Upon dispersal seeds require penetration into the ground for germination, and if exposed to direct light the seed desiccates and dies. This mechanism is likely responsible for light-inhibited *in vitro* germination of numerous species.

Van Waes and Debergh (1986b) reported that continual darkness was more beneficial for germination than a 14-hour light/10-hour dark photoperiod. Cool white fluorescent tubes provided illumination. Of 11 species examined, increased light intensity inhibited germination. For example, under continual darkness 60% germination was observed for *Orchis morio*. Light intensities of $1.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, $9 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $30.4 \mu\text{mol m}^{-2} \text{s}^{-1}$ reduced germination to 8.4%, 3.4%, and 0%, respectively.

Rasmussen et al. (1990) examined symbiotic seed germination of *Dactylorhiza majalis* incubated in darkness and then placed under a 16-hour light/8-hour dark photoperiod and vice versa. Cultures were placed under 36-watt fluorescent tubes with an irradiance of $51.2 \mu\text{mol m}^{-2} \text{s}^{-1}$. Cultures under a 16-hour light/8-hour dark photoperiod for 14 days followed by a dark incubation for 35 days increased germination (75% germination) substantially over the control (44% germination). Germination also

decreased with increased light exposure. Thirty-five days culture in darkness followed by 7 days under a 16-hour light/8-hour dark photoperiod increased germination (45% germination) from 42 days culture in 16-hour light/8-hour dark photoperiod (1.5-2.5%).

Zettler and McInnis (1994) found with *Platanthera integralabia* that a 7-day illumination period followed by a dark incubation for 70 days under both symbiotic and asymbiotic conditions lead to the greatest germination percent. Seeds in illumination were exposed to a 16-hour light/8-hour dark period under cool white fluorescent lights, and irradiance was measured at $55.8 \mu\text{mol m}^{-2} \text{s}^{-1}$. Although continuous illumination had an adverse affect on germination, a period of illumination was found to have a stimulatory effect on germination.

Rasmussen and Rasmussen (1991) examined the roles of photoperiod duration, light intensity, and light quality in the symbiotic germination of *Dactylorhiza majalis*. Cultures were subjected to continual darkness, 8-hour light/16-hour dark, and 16-hour light/8-hour dark photoperiods. Short and long-day illumination inhibited germination, while continual darkness was optimal. Cultures were also exposed to white, red, and green light. Eighteen-watt fluorescent tubes provided white light with an intensity of $60.5 \mu\text{mol m}^{-2} \text{s}^{-1}$. Red light was provided by 40-watt fluorescent lights with an intensity of $5.81 \mu\text{mol m}^{-2} \text{s}^{-1}$. To provide green light with an intensity of $0.09 \mu\text{mol m}^{-2} \text{s}^{-1}$, fluorescent tubes were covered with a gelatin filter. The low intensity of green light was inhibitory, while red light increased germination over both light types.

Table 1-1. Composition of media commonly used for *in vitro* seed culture of orchids.

Components	Knudson B Knudson, 1922	Knudson C Knudson, 1946	Curtis Medium Curtis, 1936	Malmgren Malmgren, 1996	MS Medium Murashige and Skoog, 1962
	<u>Amount (mg/L)</u>				
NH ₄ NO ₃			220		1650
(NH ₄) ₂ SO ₄	500	500			
KNO ₃					1900
Ca(NO ₃) ₂	1000	1000	350		
Ca ₃ (PO ₄) ₂				50-100	
CaCl ₂ 2H ₂ O					440
MgSO ₄ 7H ₂ O	250	250	260	50-100	370
K ₂ HPO ₄	250				
KH ₂ PO ₄		250	120	50-100	170
FePO ₄			3		
Fe ₂ (PO ₄) ₃	50				
KI					0.83
H ₃ BO ₃					6.2
MnSO ₄ 4H ₂ O		7.5			22.3
ZnSO ₄ 7H ₂ O					8.6
Na ₂ MoO ₄ 2H ₂ O					0.25
CuSO ₄ 5H ₂ O					0.025
CoCl ₂ 6H ₂ O					0.025
Na ₂ EDTA					37.3
FeSO ₄ 7H ₂ O		25			27.8
Vamin (amino acid)				300	
Soluvit (vitamins)				0.1-0.3 amp	
Dextrose			1000		
Sucrose		20000		10000	30
Agar	15000	15000	14000	6000	
Charcoal				500	

Plants of Study

Calopogon tuberosus var. *tuberosus*

Calopogon tuberosus var. *tuberosus* (L.) Britton, Sterns and Poggenberg, the common grass-pink, is a widely distributed terrestrial orchid found throughout eastern North America and several Caribbean islands (Figure 1-1). *Calopogon tuberosus* var. *tuberosus* (will be referred to as *Calopogon tuberosus*) is distributed north to Newfoundland, Canada, south to Cuba, and west to Texas (Luer, 1972). *Calopogon tuberosus* occurs throughout Florida, but is found more commonly in the north and central areas of the state (Brown, 2002; Wunderlin and Hansen, 2004) (Figure 1-1). The generic name *Calopogon*, meaning beautiful beard, is derived from the Greek words kalos (beautiful) and pogon (beard). The species name, *tuberosus*, is derived from the Latin word *tuberosus*, which inaccurately refers to the corms of the species (Luer, 1972).

Calopogon tuberosus grows in full sun and commonly inhabits acid bogs and swamps in its northern range, but in Florida common habitats include wet meadows, pine flatwoods, and sandy roadsides (Brown, 2002; Luer, 1972) (Figure 1-2). Plants readily form large populations with scattered plants, but occasionally large clusters of plants form (Figure 1-2). In a northern Michigan bog, Case (1987) observed 1,000 plants in less than one square meter. A large population in north central Florida extends approximately 0.5 miles on both sides of a county highway (P. Kauth, personal observation).

Two *C. tuberosus* varieties exist: *tuberosus* and *simpsonii* (Small) Magrath. The variety *simpsonii* is distinguished from *tuberosus* by its white floral lip, more slender leaves, taller shoots, and restricted distribution to South Florida. Variety *tuberosus* is 10-75 cm in height with 2-17 flowers per stem and 1 or 2 leaves (Case, 1987; Brown, 2002).

Individual flowers are 2-3.5 cm in diameter (Brown, 2002). The flowering period of *C. tuberosus* is March in southern Florida to August in northern states and Canada (Luer, 1975). Before plants flower, they are difficult to distinguish, due to the grass-like leaves of the species, from grass species growing in close proximity. Flowers open in slow succession, and are nonresupinate with the lip petal at the top of the plant (Luer, 1972). Flowers range in color from pale to dark pink, and white (Figure 1-3 and Figure 1-4). Keenan (1998) observed a lavender form in Maine. Plants grow from small corms, and during each successive growing season a new corm forms from the basal portion of the shoot (Figure 1-2).

Calopogon tuberosus is nectarless, and therefore relies on pollination by deceit. The lip of the flower has yellow trichomes, which resemble anthers and pollen. The column, which contains the pollen, is directly below the lip, and when pollinators land on the upper lip they fall into the column resulting in pollen deposition on the back of the insect (Firmage and Cole, 1988). Several researchers reported that *C. tuberosus* is pollinated by bumblebees including *Bombus vagans* and *B. fervidis* (Boland and Scott, 1991; Firmage and Cole, 1988).

Sacoila lanceolata* var. *lanceolata

Sacoila lanceolata (Aublet) Garay var. *lanceolata*, the leafless beaked orchid, is a terrestrial orchid distributed from North Florida to northern Uruguay (Luer, 1972) (Figure 1-5). *Sacoila lanceolata* var. *lanceolata* (will be referred to as *Sacoila lanceolata*) is distributed in Florida from the panhandle and south to Miami-Dade County (Brown, 2002; Wunderlin and Hansen, 2004) (Figure 1-5). The plants inhabit sunny roadsides and less frequently in pine flatwoods and old fields (Brown, 2002; Luer, 1972) (Figure 1-6). Since populations commonly occupy roadsides along congested highways and

interstates, the plant is listed as threatened in Florida (Brown, 2002). The generic name *Sacoila* is derived from the Greek words *saccos* (bag) and *koilos* (hollow) referring to the small spur or mentum formed by the fusion of the lateral sepals and labellum (Pridgeon et al., 2003). The species and variety name, *lanceolata*, is derived from the Latin word *lanceolatus* meaning lance-shaped, which refers to the shape of the leaves and flowers (Luer, 1972).

The flowers are a reddish color, but two flower color forms exist: *albidaviridis* the green-white form (Catling, and Sheviak, 1993) and *folsomii* the golden form (Brown, 1999) (Figure 1-6). In Florida plants flower from late April to late June. Leafless flowering stems with 10-40 flowers and 20-60 cm tall develop; however, not all plants flower every year (Luer, 1972). Luer (1972) suggested that prior to flowering plants require one or two growing seasons to accumulate nutrients (Luer, 1972). Individual flowers are approximately 2 cm in length (Figure 1-6) (Brown, 2002). After the flowering period, new leaves emerge and persist throughout summer and fall (Luer, 1972). During winter the large leaves deteriorate. Although, in Florida, plants produce flowering stems in the absence of leaves, plants in the tropics as well in cultivation flower in the presence of leaves (Luer, 1972) (Figure 1-6). The root structure of the plants is large, producing numerous thick fleshy roots (Figure 1-6).

Sacoila lanceolata is hummingbird pollinated. However, Catling (1987) reported that hummingbird pollinators are scarce in Florida. The orchid is agamospermic or apomictic by adventitious embryony, producing seed without pollination (Catling, 1987). Consequently, *S. lanceolata* populations may be in decline due to collection pressure, habitat destruction, and lack of pollinators.

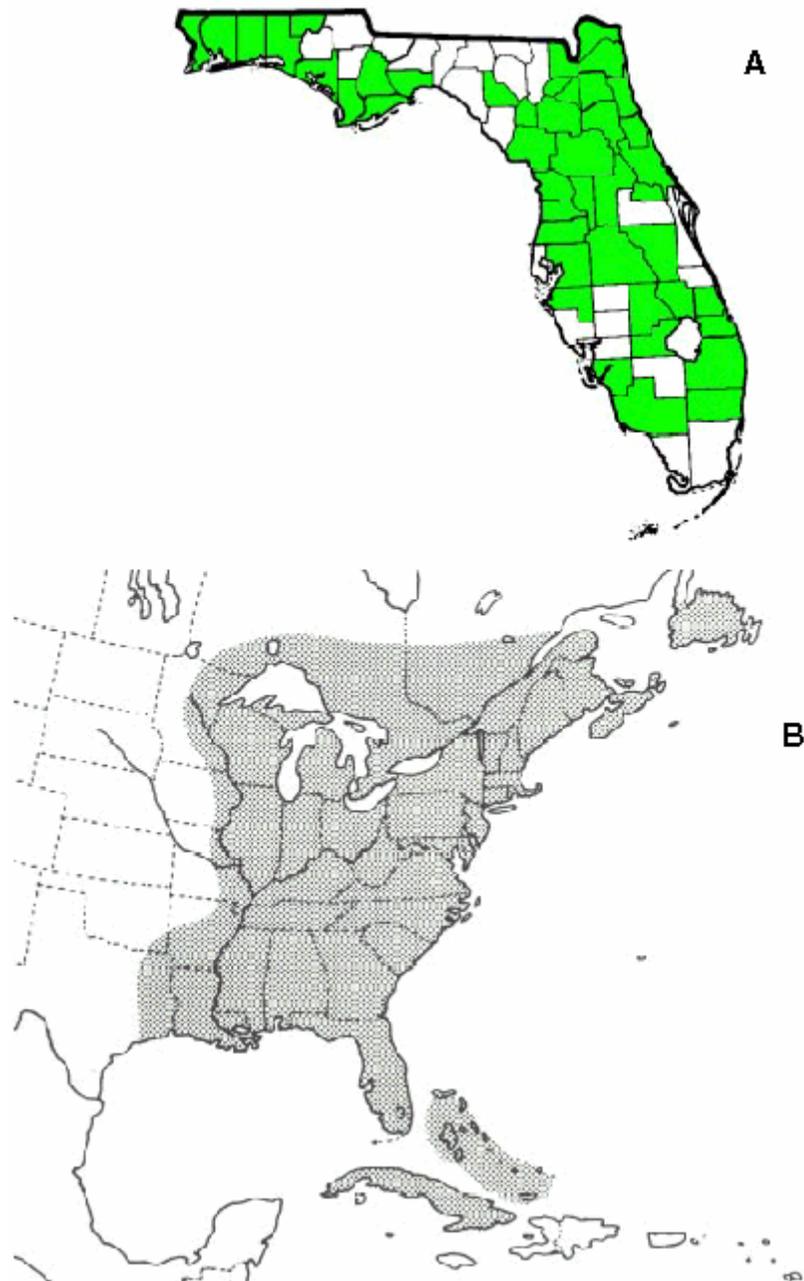


Figure 1-1. Distribution of *Calopogon tuberosus* var. *tuberosus*. A) Distribution in Florida. Green areas represent herbarium vouchers for counties where *C. tuberosus* is located. Map based on Brown (2002) and Wunderlin and Hansen (2004) B) Worldwide distribution. Darkened area represents the range of *C. tuberosus*. (From Luer, 1972 p. 58)

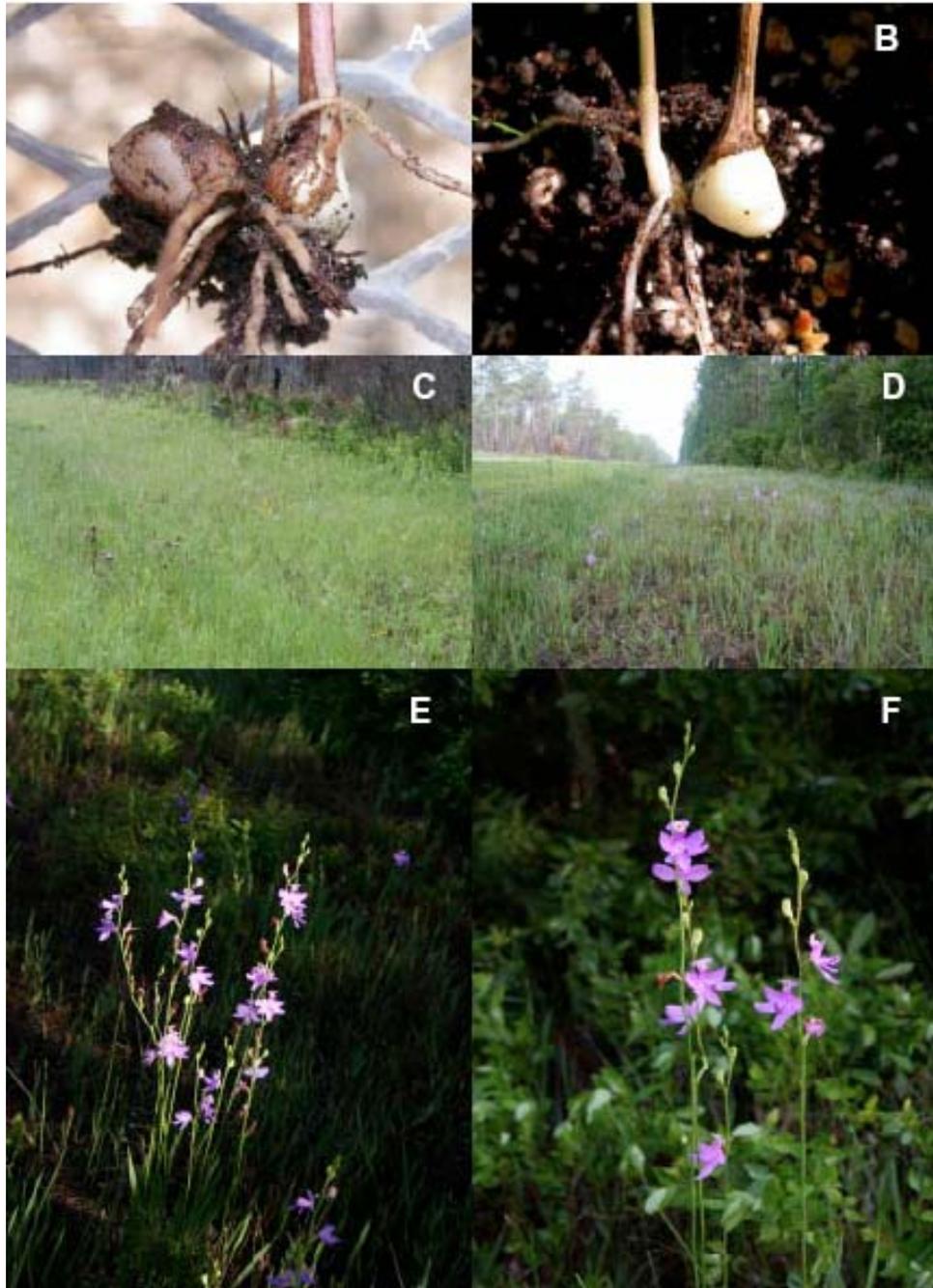


Figure 1-2. Growth habit of *Calopogon tuberosus*. A) Old corm (left) from previous growing season and new corm with shoot (right) from new season of growth. B) Young shoot with no corm (left) and old corm from previous growing season. C) Roadside habitat near Bryceville, Nassau County, Florida. D) Roadside habitat in Goethe State Forest, Levy County, Florida. E) Large group of plants in Goethe State Forest, Levy County, Florida. F) Up-close view of a group of plants in Goethe State Forest, Levy County, Florida.

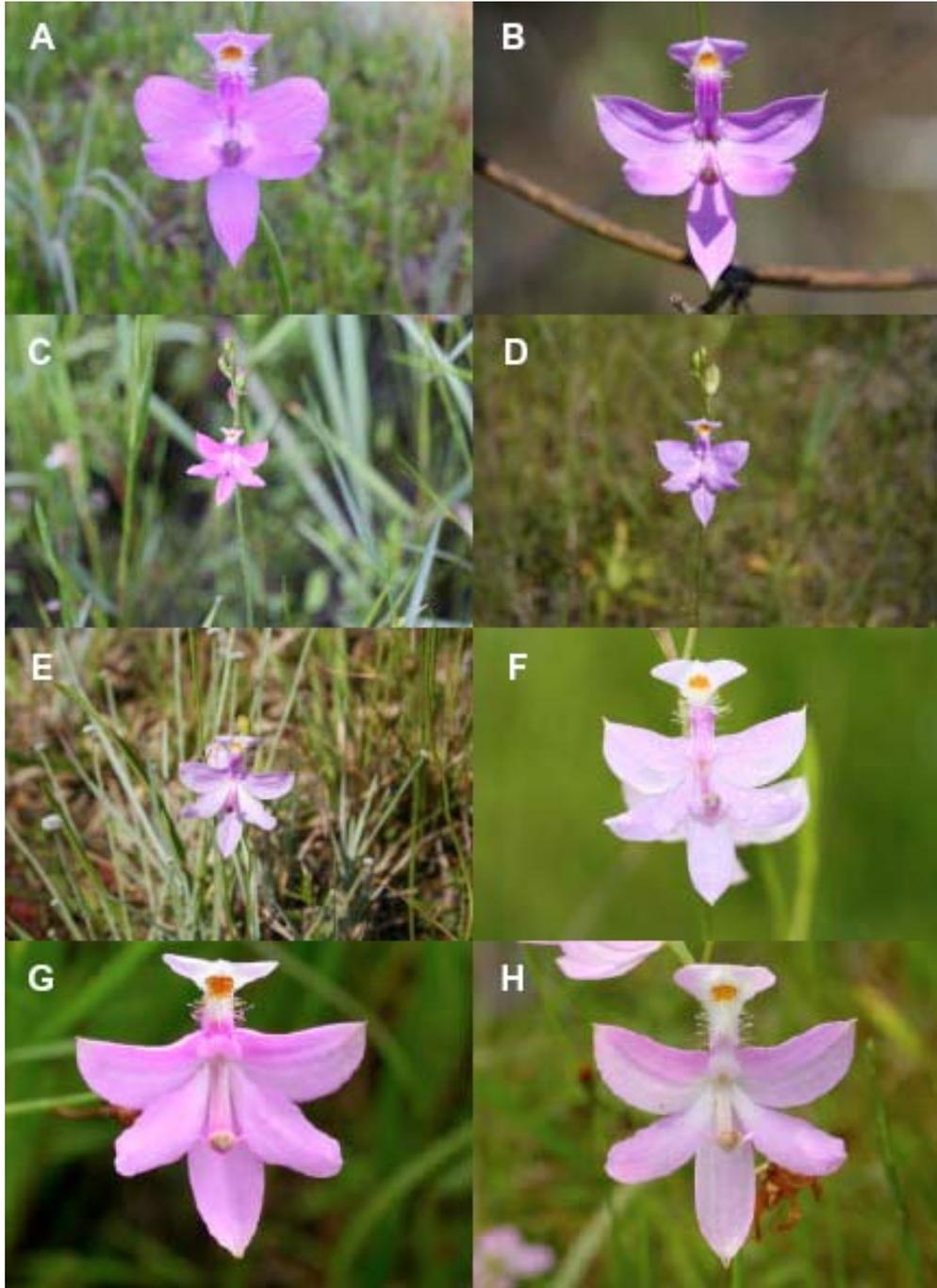


Figure 1-3. Various pink flower forms of *Calopogon tuberosus*. Plants located in Goethe State Forest, Levy County, Florida. A&B) Typical pink/purple color form. C) Darker pink color form. D&E) Lighter pink/purple color form. F) Typical light pink-to-white color form. G) Pink color form with lighter upper lip color. H) Unusual pink/white color form.

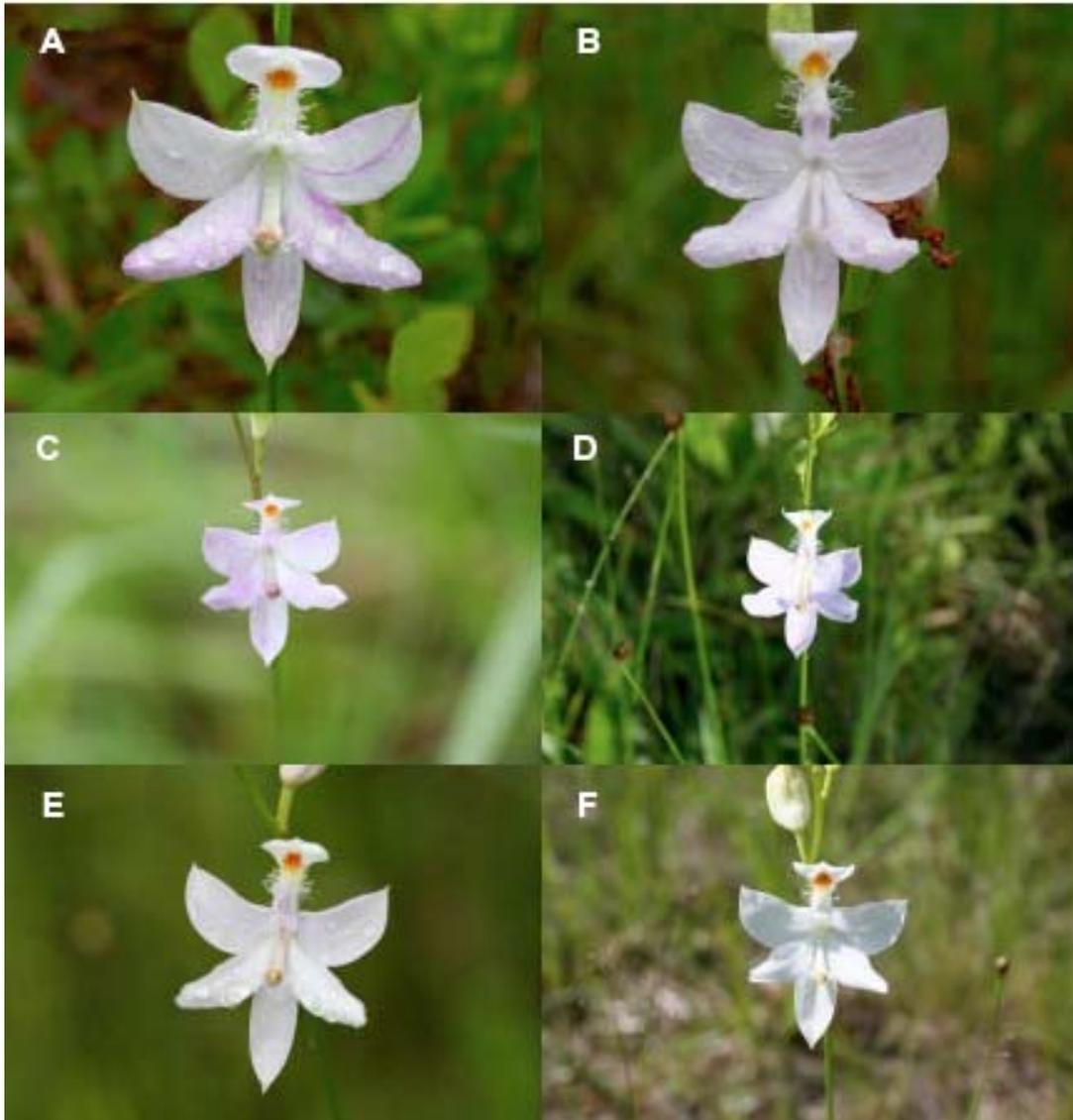


Figure 1-4. White flower color of *Calopogon tuberosus*. Plants growing in Goethe State Forest, Levy County, Florida, and near Bryceville, Nassau County, Florida. A) Rare flower color with several splashes and stripes of purple/pink. B-E) Various degrees of white with a hint of pink. F) *Calopogon tuberosus* var. *tuberosus* forma *albiflorus*-pure white color form.

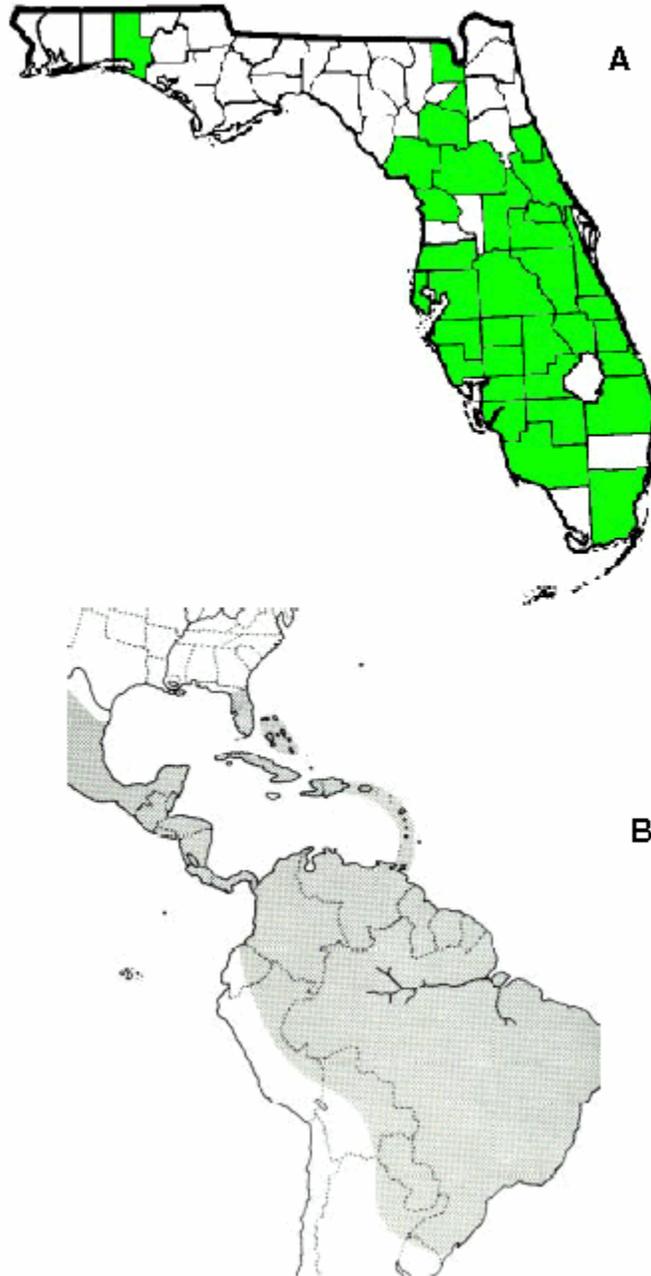


Figure 1-5. Distribution of *Sacoila lanceolata* var. *lanceolata*. A) Distribution in Florida. Light green areas represent herbarium vouchers for counties where *S. lanceolata* is located. Map based on Brown (2002) and Wunderlin and Hansen (2004) B) Worldwide distribution. Dark areas represent the range of *S. lanceolata* (From Luer, 1972 p. 118)

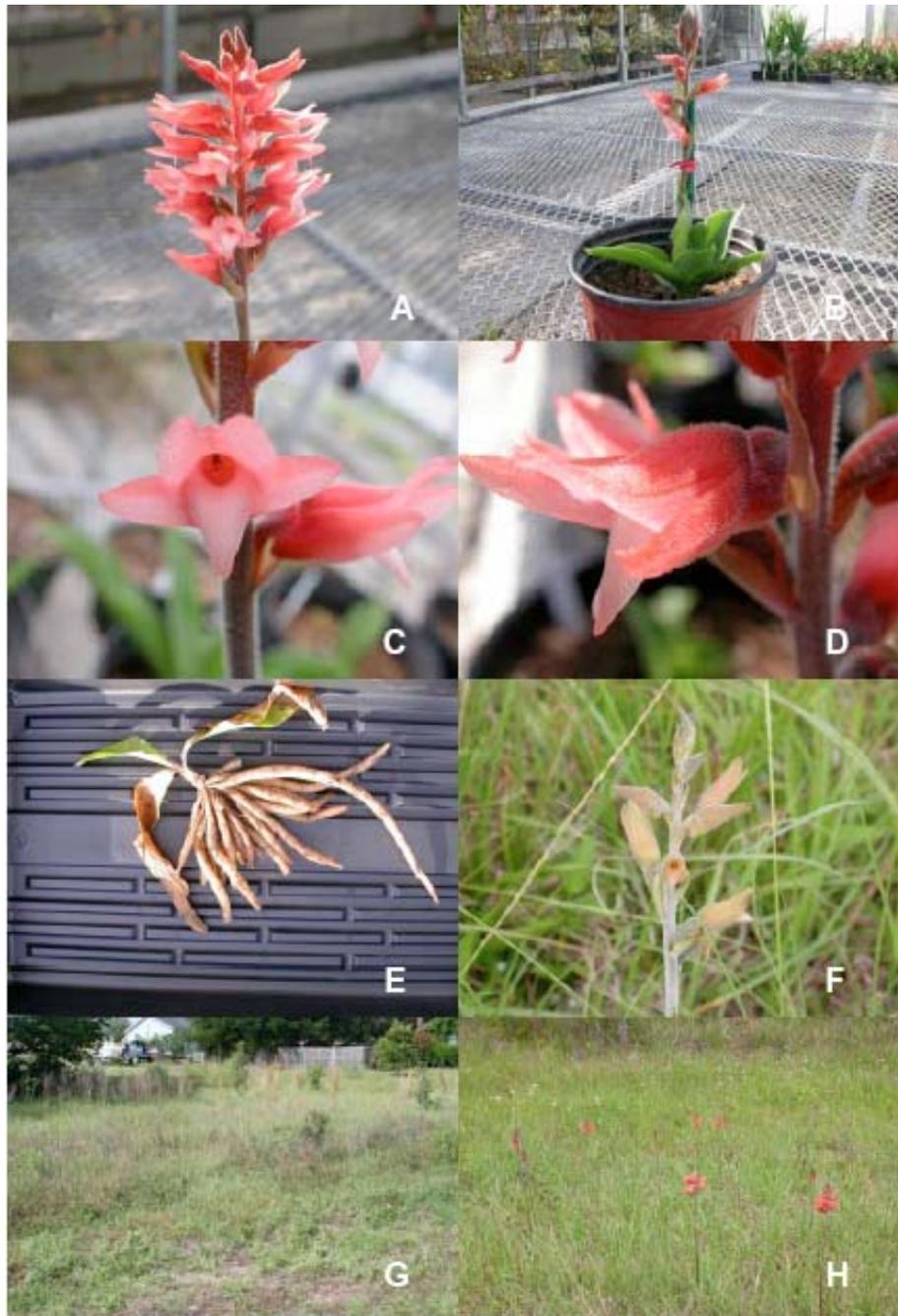


Figure 1-6. Growth habit of *Sacoila lanceolata* var. *lanceolata*. A) Inflorescence with approximately 30 individual red flowers. B) Cultivated plant flowering in presence of leaves. C&D) Upclose frontal view and profile of an individual flower. E) Large fleshy root structure. F) *Sacoila lanceolata* var. *lanceolata* forma *folsomii*, the golden yellow color form, on Florida's turnpike. G) Field habitat with 20-30 flowering plants near Dunnellon, Levy County, Florida. H) Roadside habitat in Goethe State Forest, Levy County, Florida. Note that plants in nature flower in the absence of leaves.

Seed Culture of *Calopogon tuberosus*

Calopogon tuberosus is one of the most widespread orchids of eastern North America, thus published research is fairly prevalent. Since *C. tuberosus* is a widespread, showy species, several small commercial nurseries offer both species and hybrids. Recently, much of the research has focused on the systematics and taxonomy among the five species of *Calopogon* (Goldman et al., 2004a; Goldman, et al., 2004b; Trapnell et al., 2004). Although studies are common for seed culture of *C. tuberosus*, the techniques outlined are contradictory to each other, and research is needed to properly compare these factors.

In an early study on seed culture of *C. tuberosus* (referred to as *C. pulchellus*), Liddell (1944) reported success using a liquid Knudson B solution rather than solidified agar medium. Within one month, seeds germinated and protocorms formed leaves. Seedlings were transferred to Knudson solidified medium after leaves began growing. Within 4 months seedlings reached a height of 5 cm.

Stoutamire reported the seed culture of terrestrial species including *C. tuberosus* using Knudson C medium (Stoutamire, 1964, 1974, 1983). Stoutamire (1964) also observed that seeds of *C. tuberosus* became photosynthetic in sterile distilled water within 3 weeks. Seeds were cultured on Knudson C under natural light (north-facing windows) and fluorescent lights (12 hour light/12 hour dark photoperiod) at 20 C. Embryos turned green within 2 weeks after inoculation and developed the first leaf within 4 months. The shoot then senesced, and seedlings became dormant. To break shoot dormancy, seedlings were vernalized for 4-6 weeks. Completion of the growth cycle required 3-5 months, and *C. tuberosus* required a total of 41 months to flower.

Carson Whitlow, owner of Cyp. Haven (Adel, Iowa), a small commercial orchid nursery, has outlined a commercial seed culture protocol for *C. tuberosus* (Whitlow, 1996). Only mature seeds were used, and no one specific culture medium provided superior results (Whitlow, pers. comm.). Seeds were first inoculated onto culture medium (no mention of which medium), and quickly grew to about 3 cm. All cultures were placed 15 cm below fluorescent lights. After 6 months culture, seedlings became dormant. Seedlings were then vernalized in darkness for 3 months at 4 C, after which cultures were again placed under fluorescent lights. After 5 to 6 months under light, plants were vernalized again under the same conditions, and following vernalization seedlings were planted in a 1:1 mixture of sand and peat moss.

While Whitlow used only mature seeds, others have used immature seeds for *C. tuberosus* (Arditti et al., 1985; Riley, 1983). Arditti et al. (1985) germinated immature seeds on Curtis medium. Green protocorms formed 10-14 days after initiation of seed germination, nearly 100% germination was achieved after 2 months, and plantlets 5-10 cm in height developed after 2-4 months. Riley (1983) found that using near mature seeds of *C. tuberosus* yielded higher germination rates than immature seeds and fully mature seeds, and a modified Lucke O4 medium (Lucke, 1971) yielded superior germination rates. The developing seedlings were vernalized at 4 C for several months.

Henrich et al. (1981) and Myers and Ascher (1982) used mature seeds of *C. tuberosus*. Both research teams used a medium developed by Norstog (1973) for the culture of barley embryos. Henrich et al. (1981) stored cultures in polyethylene bags in continuous darkness at 25 C for 6 months. After 29 days, total germination was 25%. Myers and Ascher (1982) also stored cultures in the dark at 25 C. After protocorms

developed, they were transferred to MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.6% agar, and 0.2% activated charcoal and stored in dark at 25 C. When leaf expansion occurred, seedlings were transferred to MS medium and placed under cool white fluorescent lights (16 hour light/8 hour dark) with an intensity of $160 \mu\text{mol m}^{-2}\text{s}^{-1}$ for 1-3 weeks. When at least one leaf fully expanded and dark green roots formed, plantlets were removed from culture and potted in sphagnum moss. No data were reported for greenhouse culture.

Anderson (1990) also used mature *C. tuberosus* seeds, which were air-dried for 48 hours and stored at 4 C with anhydrous calcium chloride in order to absorb excess moisture. Cultures were placed in both light and darkness at 22 C (no reference to light source or intensity). A comparison was made between Burgeff N₃f (Burgeff, 1936) medium, Knudson C medium, and a modified Lucke medium developed by the author. Leaf elongation occurred fastest on Lucke medium compared to the other culture media. Five cm leaves developed in 8 weeks on Lucke medium, and seedlings maintained under fluorescent lights were larger than those cultured in darkness. Within 2 months, light-grown seedlings produced small corms, 5 cm long leaves, and roots. Unlike Whitlow (1996), who allowed the seedlings to end their growth cycle *in vitro*, 2 month-old seedlings were planted in a potting mix. However, data were not provided on the greenhouse culture of the species.

Based on a review of the published literature, information on specific cultural conditions for optimum seed germination and seedling development for *C. tuberosus* and *S. lanceolata* are limited. This information is essential for development of commercially viable propagation protocols. The overall objectives of this research are to establish

protocols for *in vitro* seed culture, as well as study seedling development of *C. tuberosus* and *S. lanceolata*.

CHAPTER 2
SEED CULTURE AND SEEDLING DEVELOPMENT OF *Calopogon tuberosus*

Introduction

The Orchidaceae, the largest plant family with 20,000 to 30,000 species, comprises 10% of all angiosperms (Cronquist, 1981; Dressler, 1981). Recently, orchids such as *Phalaenopsis* hybrids have become popular houseplants. Although no statistics are available, several researchers have mentioned that *Phalaenopsis* orchids make up 75 to 90% of all orchid sales (Griesbach, 2002; Nash, 2003). Currently orchids are the second most popular potted floriculture crop behind poinsettias. Wholesale orchid sales in 2003 were estimated at approximately \$122 million and increased to \$128 million in 2004 (USDA, 2004).

Although orchid sales are quickly rising, production and sales of native orchids, at best, are slowly increasing. Production of native orchids has not been fully commercialized, but is centralized within hobby growers and small, specialized nurseries. These nurseries only offer a small selection of showy genera and species. The small market for native terrestrial orchids is influenced by a lack of knowledge from the consumer, lack of interest by the industry, difficulties in propagation methods, and the long time period required to obtain flowering plants.

A native orchid with horticultural potential is *Calopogon tuberosus* (L.) Britton, Sterns and Poggenberg, the common grasspink. *Calopogon tuberosus*, a widespread terrestrial orchid of eastern North American, is characterized by grass-like leaves and small pink flowers. Flower color can vary from white, lavender, to pale and dark pink

(Figure 1-3 and Figure 1-4) (Luer, 1972). Recently artificial hybrids have been commercially available such as *Calopogon* 'Fluffy', a cross between *C. tuberosus* and *C. multiflorus*.

Since *C. tuberosus* is both widespread and common, research is abundant. Ecological observations began as early as the late 1800s (Robertson, 1887) with seed culture studies beginning in the early to mid 1900s (Carlson, 1936). Researchers as early as Carlson (1936) have stated that *C. tuberosus* germinates quickly under *in vitro* conditions. Recent advances in seed culture and breeding of *C. tuberosus* have led to increased production, and *C. tuberosus* is quickly becoming a popular garden orchid. Although seed culture has been studied extensively, much of the results generated are contradictory and incomplete.

Terrestrial orchids are suited for survival in variable habitats, and optimal *in vitro* culture conditions suitable for one species or genotype may differ from others (Van Waes and Debergh, 1986b). One such culture condition is photoperiod. Debate exists whether complete darkness or a period of illumination is optimal for the germination of *C. tuberosus* seeds. Whitlow (1996) incubated cultures under cool white fluorescent lights and achieved a high germination rate. Anderson (1990) compared germination between complete darkness and illumination, and found that illuminated seedlings developed quicker and became larger than those under complete darkness. Henrich et al. (1981) and Myers and Ascher (1982) incubated cultures under complete darkness and found that seeds germinated quickly.

Although no medium was specifically described, Whitlow (1996) stated that seeds of *C. tuberosus* germinate equally on several media. Several media reported in published

studies include Curtis medium (Curtis, 1936), Lucke O4 medium (Lucke, 1971), Knudson C (Knudson, 1946), and MS (Murashige and Skoog, 1962). Recently, hobbyists have developed media specifically for terrestrial orchids such as that formulated by Malmgren (1996). However, media developed by hobbyists are rarely used in scientific studies.

This study was undertaken to develop an efficient seed culture protocol as well as examine seedling development by exploring the role of photoperiod and culture media. Terrestrial orchids have been reported to mature and flower slowly (Stoutamire, 1964, 1974). This long maturation process negatively impacts growers by preventing the sales of mature plants. An efficient seed culture protocol may generate a larger number of mature seedlings more quickly than currently available seed culture protocols.

Materials and Methods

Mature seeds of *Calopogon tuberosus* were collected from Goethe State Forest, Levy County, Florida in July and August 2004. Seeds were removed from capsules, pooled, and stored in scintillation vials at 21-23 C in a desiccator with anhydrous calcium sulfate to absorb excess moisture. Seeds were surface-sterilized for 2 minutes in sterile scintillation vials containing 8 ml of an aqueous solution consisting of 0.33% sodium hypochlorite, 5% ethanol (100%), and 90 ml sterile distilled deionized water. Following surface-sterilization, seeds were rinsed three times for 2 minutes each in sterile distilled deionized water. Solutions were drawn out of the scintillation vial with a disposable 1000 µl sterile pipet tip that was replaced after each use. Seeds were then suspended in sterile deionized distilled water, and a sterile inoculating loop was used for inoculating culture vessels. The inoculating loop was immersed once into the seed suspension and

seeds were placed in the culture vessel. The average number of seeds per individual inoculation was approximately 93 ± 27 .

Media Preparation

Three basal media (Table 2-1 and Table 2-2) commercially prepared by *Phytotechnology Laboratories, L.L.C* (Shawnee Mission, KS) were assessed for germination efficiency: Malmgren modified terrestrial orchid medium (MM) (from Malmgren, 1996), modified Knudson C (KC) (from Knudson, 1946), and *Phytotechnology* orchid seed sowing medium (catalog number P723). Malmgren medium with no pineapple powder was special ordered from *Phytotechnology Laboratories*. Malmgren and KC were standardized to P723 by the addition of 2% sucrose and 0.8% TC® agar (*Phytotechnology, Shawnee Mission, KS*) as well as 0.1% charcoal to KC. Media were adjusted to pH 5.7 with 0.1 N KOH before the addition of agar and sterilization for 40 minutes at 1.2 kg cm^{-2} and 121 C.

Sterile media were dispensed as 50 ml aliquots into square 100 x 15 mm petri dishes (Falcon “Integrid” Petri Dishes, Becton Dickinson, Woburn, MA). The bottom of each petri dish was divided into 36, 13 x 13 mm cells. Of the 36 cells, the interior 16 were used for seed inoculation to avoid media desiccation at the edges of the petri dish. Five of the 16 interior cells were randomly selected for seed inoculation using a computerized random number generator. Ten replicate petri dishes were inoculated per treatment. Petri dishes were sealed with a single layer of Nescofilm (*Karlan Research Products, Santa Rosa, CA*) to avoid desiccation.

Light Treatments

Effects of five light treatments were evaluated: 1) 8 weeks in a 16 hour light/8 hour dark photoperiod; 2) 8 weeks continual darkness; 3) 2 weeks darkness followed by 6

weeks under a 16 hour light/8 hour dark photoperiod; 4) 4 weeks darkness followed by 4 weeks under a 16 hour light/8 hour dark photoperiod; 5) 6 weeks darkness followed by 2 weeks under a 16 hour light/8 hour dark photoperiod. Light was provided by GE F96T12 fluorescent lights at an average of $91.6 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured at culture level and 25 ± 2 C. Cultures in the dark treatment were placed in a light-free cabinet at 24.2 ± 0.6 C. Cultures maintained under 8-week 16-hr photoperiod were evaluated weekly for 8 weeks to determine percent germination. After culture in 2 weeks, 4 weeks, and 6 weeks dark, cultures were placed under previously described light conditions and evaluated weekly until week 8. Seeds cultured in the 8-week dark period were evaluated once at week 8. Seedling development was evaluated weekly using the six stages of seedling development (Table 2-3) modified from Stenberg and Kane (1998). Cultures were viewed under a Nikon SMZ100 Stereoscope (Southern Micro Instruments, Marietta, GA) equipped with a Nikon Coolpix 4500 (Nikon Corporation, Tokyo, Japan).

Seedling Development

After 16 weeks culture, seedlings in each treatment were transferred to *Phytotech* Culture Boxes (95 mm x 95 mm x 110 mm) containing 100 ml of corresponding media (Figure 2-16). Seedlings from each photoperiod treatment were maintained separately. Fifteen seedlings were transferred to each vessel. After 24 weeks total culture, 50 seedlings from each treatment combination (media x photoperiod) were randomly selected for evaluation of leaf length and number, root length and number, fresh weight, and dry weight. The longest root and leaf were measured. Seedlings were placed in a drying oven for 48 hours at approximately 60 C to measure dry weight.

Table 2-1. Formulations of orchid seed sowing medium (P723), Malmgren modified terrestrial orchid medium (MM), and Modified Knudson C (KC) medium as prepared by *Phytotechnology Laboratories*.

Component	Amount/Medium (mg/L)		
	P723	MM	KC
Ammonium Nitrate	412.5		500
Ammonium Sulfate			500
Boric Acid	1.65		
Calcium Chloride	83		
Calcium Nitrate			347.2
Calcium Phosphate, tribasic	75		
Cobalt Chloride · 6H ₂ O	0.0063		
Cupric Sulfate · 5H ₂ O	0.0063		
Na ₂ EDTA	18.25	37.26	
Ferrous Sulfate · 7H ₂ O	13.93	27.8	25
Magnesium Sulfate	75.18	97.69	122.13
Manganese Sulfate · H ₂ O	4.23	1.54	5.682
Molybdic Acid · 2H ₂ O	0.0625		
Potassium Chloride			250
Potassium Iodide	0.2075		
Potassium Nitrate	475		
Potassium Phosphate, monobasic	42.5	75	250
Zinc Sulfate · 7H ₂ O	2.65		
Casein		400	
D-Biotin		0.05	
Folic acid		0.5	
Glycine		2	
MES (Free Acid)	500		
myo-inositol	100	100	
Nicotinic acid	1		
Peptone	2000		
Pyridoxine HCl	1	1	
Thiamine HCl	10	10	
Charcoal	1000	1000	
Agar	8000	8000	8000
Sucrose	20000	20000	20000

Source: *Phytotechnology Laboratories Catalogue & Laboratory Guide 2004-2006*.

Table 2-2. Comparative mineral salt content of orchid seed sowing medium (P723), Malmgren modified terrestrial orchid medium (MM), and Knudson C (KC) as prepared by *Phytotechnology* Laboratories.

	P723	MM	KC
<u>Macronutrients (mM)</u>			
Ammonium	5.15		10.03
Calcium	0.75	0.24	2.12
Chlorine	0.75		3.35
Magnesium	0.62	0.81	1.01
Nitrate	9.85		8.37
Potassium	5.01	0.55	5.19
Phosphate	0.31	0.55	1.84
Sulfate	0.08	0.10	4.91
<u>Micronutrients (µM)</u>			
Boron	26.68		
Cobalt	0.03		
Copper	0.03		
Iron	50.10	99.99	89.90
Iodine	1.25		
Manganese	25.00	9.12	33.60
Molybdenum	0.26		
Zinc	9.22		
Total N (mM)	15.00		18.40
NH ₄ ⁺ :NO ₃ ⁻	0.52		1.12
Total mM	22.64	2.36	36.93

Source: *Phytotechnology* Laboratories Catalogue and Laboratory Guide 2004-2006.

Table 2-3. Six stages of orchid seed development.

Stage	Description
1	Imbibed seed, swollen and greening still covered or partially covered by testa
2	Enlarged seed without testa
3	Protocorm with pointed shoot apex and rhizoids
4	Protocorm with developing leaves and rhizoids
5	Seedling with one or more leaves and one or less developing roots
6	Seedling with evident roots and two or more leaves

Adapted from Stenberg and Kane, 1998, adapted from Mariat, 1952.

After 7 months culture, large corms from seedlings cultured on P723 were transferred to *Phytotech* Culture Boxes containing 100 ml of fresh P723. Due to shoot senescence, shoots were removed from corms. A total of 20 culture vessels were inoculated with 12 corms per vessel. Culture vessels were placed in cold-storage at 10.9 ± 0.17 C for either 1 or 2 months. Ten cultures were placed in each time period. After cold-storage, culture vessels were placed under previously described light conditions, and observations were recorded on new growth of corms.

Acclimatization Study

Potting media effects on seedling *ex vitro* acclimatization were studied. Four potting media were examined: sphagnum moss (Chile), vermiculite, Fafard mix 2 (Conrad Fafard, Inc., Agawam, MA) and a 1:1 (v/v) peat moss/sand mixture. Fafard mix 2, a commercially available mix, consists of 55% Canadian peat and equal parts perlite and vermiculite. Potting media were randomly assigned to nine plugs (5.08 cm diameter x 6.35 cm tall) in a 38-cell plug tray. Eight replicate plug trays were used. Only seedlings cultured on P723 in the initial 8-week 16-hr photoperiod were used for the experiment. One randomly selected 6 month-old seedling was placed in each plug cell. Seedlings were acclimatized from March through May 2005.

Plug trays were covered with clear vinyl humidity domes to prevent desiccation and placed under 50% shade cloth in the greenhouse with an average light level of $795 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured at noon. After 2 weeks domes were partially removed and completely removed one week later. Seedlings were watered weekly while under humidity domes and 3-4 days after. After one-month acclimatization, seedlings were fertilized weekly with 150 ppm N-P-K balanced liquid fertilizer (Peter's 20-20-20, The Scott's Company, Marysville, OH). Average temperatures ranged from $21.6 \pm 2.0 \text{ C}$ to $28.8 \pm 3.0 \text{ C}$, and humidity levels ranged from 51 to 97%. After 12 weeks, seedlings were examined for growth and development including leaf length and number, root length and number, fresh and dry weight, corm diameter, and survivorship. Lengths were calculated by measuring the longest leaf and root, and diameters were measured at the widest portion of the corm.

Statistical Analysis

Germination rates were calculated by dividing the number of seeds in stages 1-6 by the total number of seeds in the subsample. The percentage of protocorms and seedlings in a developmental stage was calculated by dividing the number of seeds in that stage by the total number of germinated seeds. Seed germination, seedling development, and acclimatization data were analyzed using general linear model procedures, least square means, and LSD at $\alpha=0.05$ in SAS v 8.02. Germination counts were arcsine transformed to normalize the variation.

Results

Effect of Light Treatment and Culture Media

Reports have indicated that seed germination of terrestrial orchids increases under complete darkness compared to illumination (Oliva and Arditti, 1984; Van Waes and Debergh, 1986b). This experiment was performed to study the effects of dark and light as well as culture medium type on seed germination. Seeds became swollen quickly after inoculation onto culture media, and germination commenced within the first two weeks of culture. The overall visual contamination rate of cultures was approximately 13%. A tetrazolium test of *C. tuberosus* seeds from the Goethe State Forest population yielded approximately 35% viable embryos (S. Stewart, unpublished). Among all light and media treatments, seed germination was significantly higher on KC (Figure 2-1). Lowest germination occurred on KC in the three pretreatments of 2 weeks, 4 weeks, and 6 weeks dark (Figure 2-1).

Seed germination on MM in 8 weeks dark was significantly higher than germination on MM in an 8-week 16-hr photoperiod (Figure 2-1). Seed germination on P723 in the 8-week 16-hr photoperiod was significantly higher than seed germination on P723 in 8 weeks dark. In the 8-week 16-hr photoperiod, no significant difference in germination was found between MM and P723, however; seed germination was significantly higher on KC.

Seed germination on all media incubated in an 8-week 16-hr photoperiod commenced by week one (Figure 2-2). Throughout the initial 8-week culture period in light, maximum germination occurred at 4, 5 and 6 weeks culture on KC, P723, and MM, respectively. After germination peaked, percent germination either stayed the same or decreased slightly due to embryo, protocorm, and seedling death.

Although seed germination was highest in complete darkness on KC, subsequent seedling development was not enhanced on this medium (Figure 2-3). Total seed germination on P723 was lower compared to KC and MM, but seed development was more rapid. At week 8 total seed germination on P723 in the 8-week 16-hr photoperiod was 33%. Of this 33%, nearly 28% of the germinated seeds developed to Stages 5 and 6. On KC cultured in the 8-week 16-hr photoperiod, 22% of the 42% germinated seeds developed to Stages 5 and 6. Seedlings developed to Stages 5 and 6 slowly on MM in the 8-week 16-hr photoperiod with only 9% of the 32% germinated seeds exhibiting advanced development.

Seeds incubated in complete darkness did not develop to Stage 6 on any media. A small percentage of seedlings incubated in complete darkness developed to Stage 5. Approximately 3% of germinated seeds developed to Stage 5 on all media in complete darkness. Although Stage 5 development was low in complete darkness, a higher percentage of seedlings cultured on MM in complete darkness developed to Stages 3 and 4 than on MM in the 8-week 16-hr photoperiod (Figure 2-3).

With respect to developmental time course, seedlings in the 8-week 16-hr photoperiod cultured on P723 developed more rapidly than those on KC or MM (Figure 2-4). On P723 seedlings developed to Stage 6 after week 5, while those on MM and KC developed to Stage 6 only after week 7. Seedlings developed to Stage 5 after week 5 on KC and MM and week 2 on P723. On MM and KC at week 8 the greatest percentage of seedlings developed to Stage 1 and 3, respectively. At week 8 on P723, the highest number of seedlings developed to Stage 6. The previous results show that seedling development was enhanced by light incubation on P723.

Several reports have indicated that darkness before light inhibited germination (Rasmussen, 1992; Zettler and McInnis, 1994). In this study dark pretreatments preceded illumination ($91.6 \mu\text{mol m}^{-2} \text{s}^{-1}$) in an 8-week 16-hr photoperiod. The pretreatments included 2 weeks dark, 4 weeks dark, and 6 weeks dark. Seed germination was significantly lower in the dark pretreatments than 8-week dark and 8-week light periods (Figure 2-1). Between the three dark pretreatments seed germination on KC was significantly lower than MM and P723 (Figure 2-1). Among seeds germinated on P723, seed germination was significantly higher for those cultured for a 6-week dark period. Among seeds germinated on MM, germination was highest in 4 weeks dark pretreatment.

Over time seed germination decreased in 2 weeks dark, generally increased or stayed the same in 4 weeks dark, and stayed the same in 6 weeks dark (Figure 2-5). Seed germination on P723 generally decreased over time in all light treatments, while seed germination on KC and MM either stayed the same or increased slightly (Figure 2-5). Seeds cultured on P723 developed to more advanced stages than those on KC and MM (Figure 2-6). Only seeds germinated on P723 developed to Stage 5. MM proved to be the least productive for seedling development as the majority of seeds developed to Stages 1 and 2 (Figure 2-6).

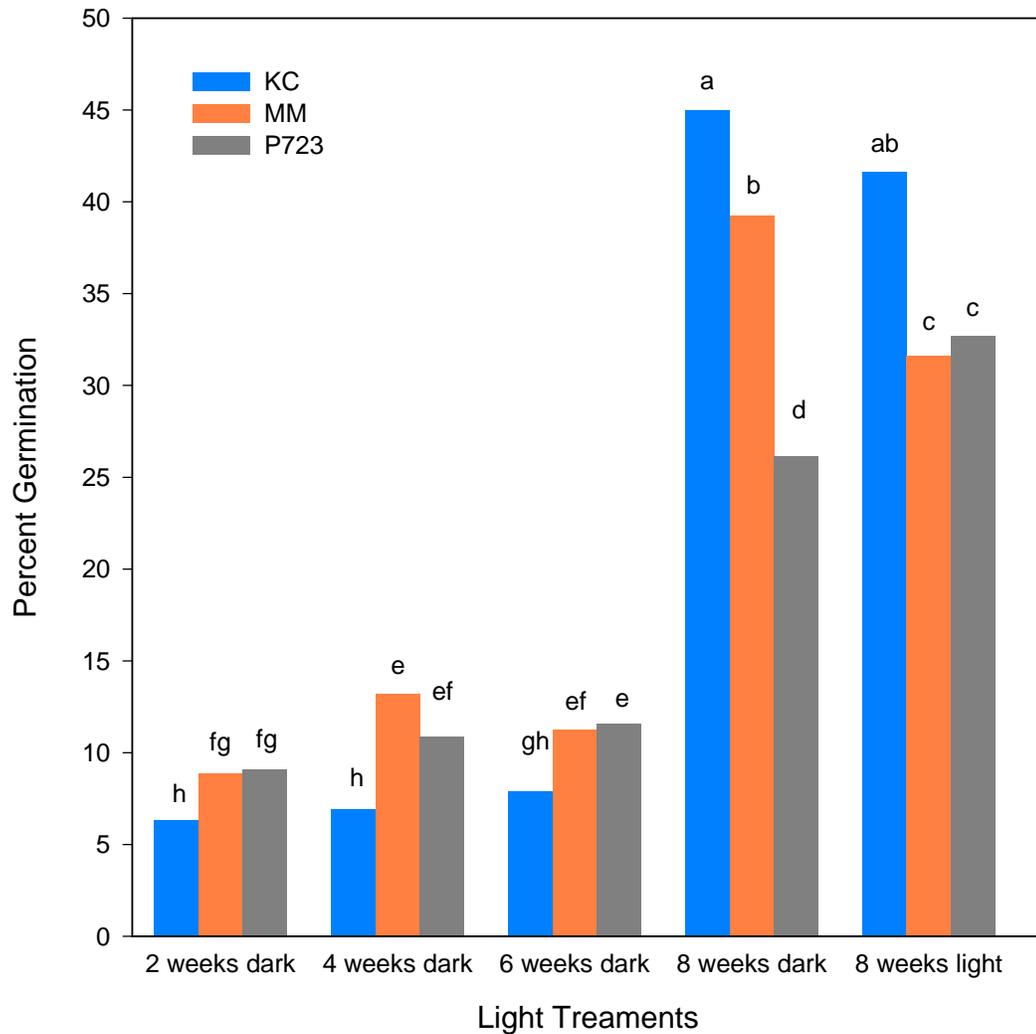


Figure 2-1. Effect of culture media and light treatment on percent seed germination of *Calopogon tuberosus* after 8 weeks culture. Histograms represent the mean response of 10 replicate plates each with 5 subsamples. Histograms with the same letter are not significantly different across light treatments ($\alpha=0.05$). KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium.

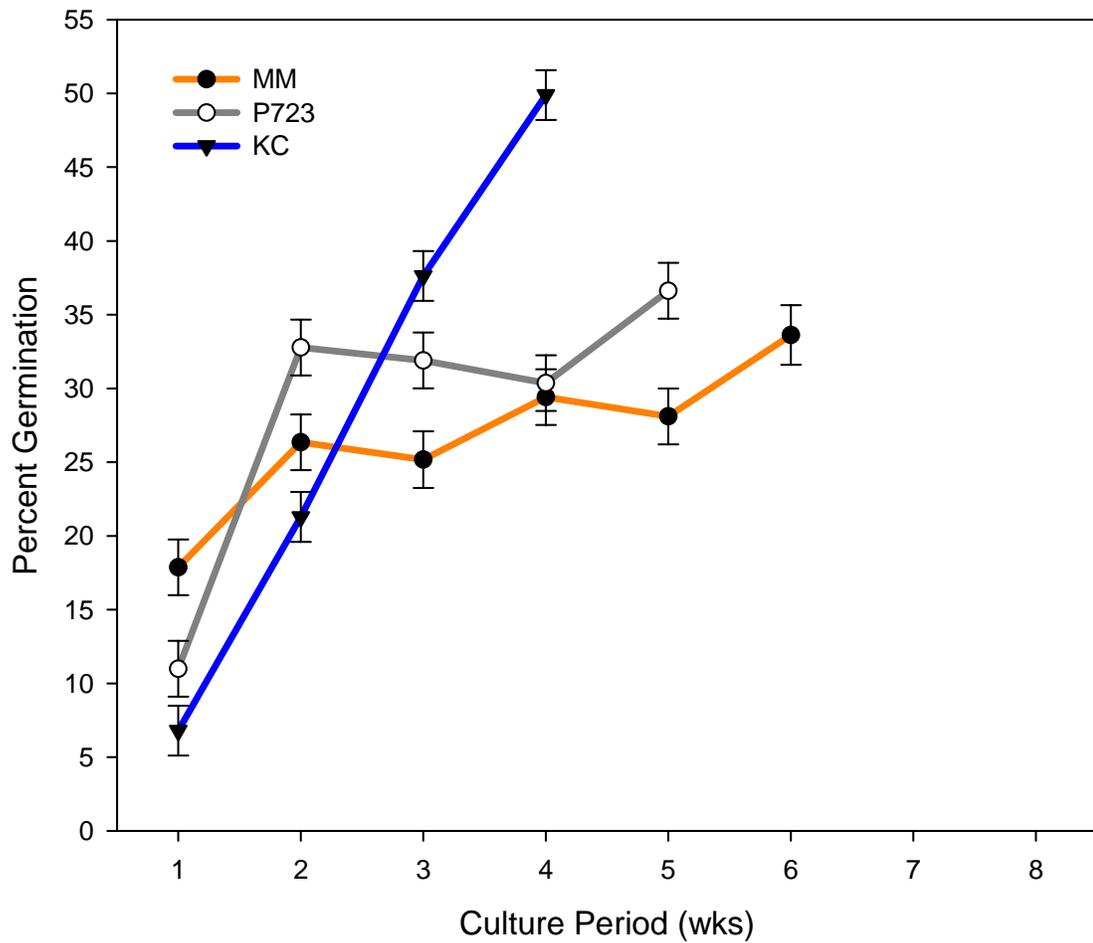


Figure 2-2. Effect of culture media on maximum germination rates of *Calopogon tuberosus*. Seeds were cultured in a 16-hour light/8-hour dark photoperiod. Each point represents the mean response (\pm S.E.) of 10 replicate plates each with 5 subsamples. KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium.

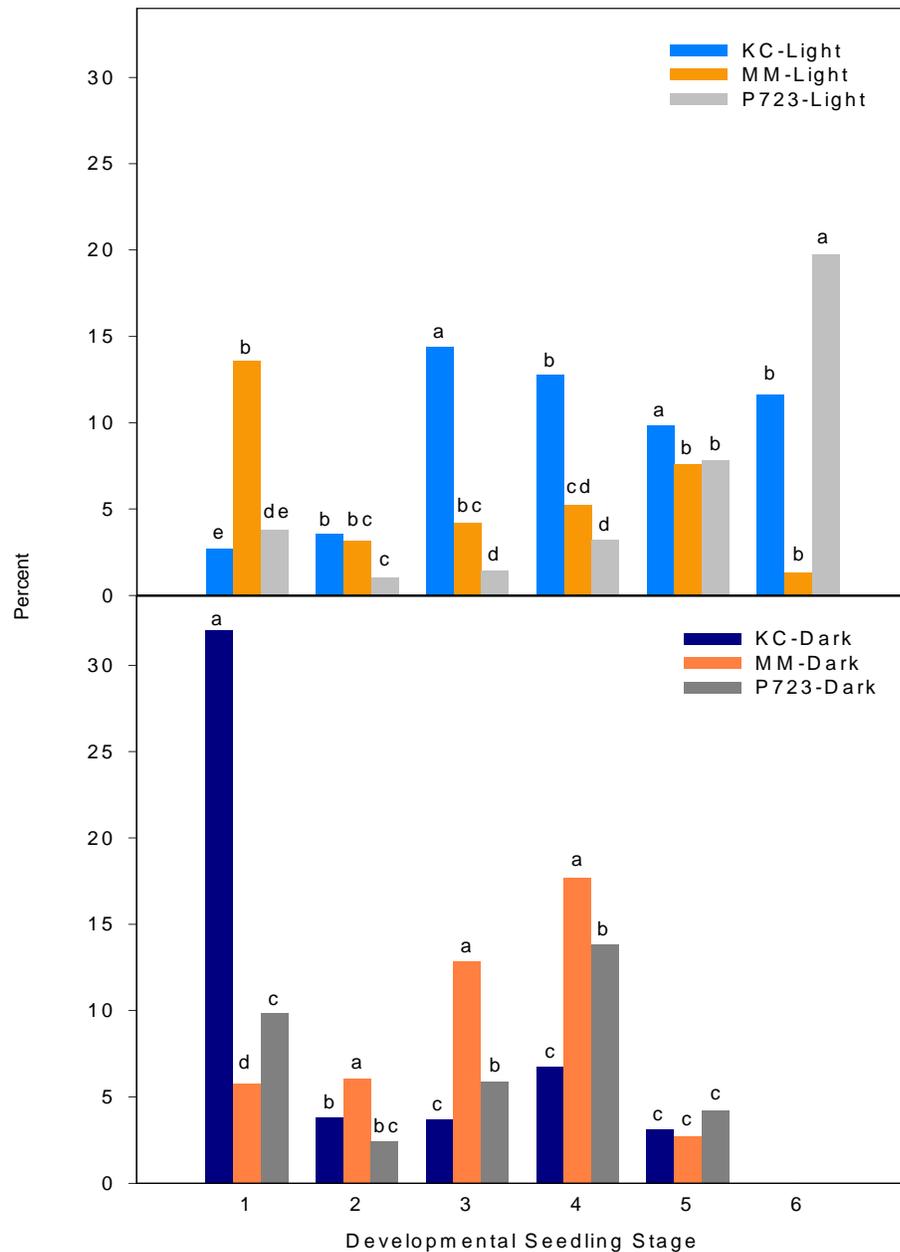


Figure 2-3. Effect of light treatment and culture media on *in vitro* development of *Calopogon tuberosus* protocorms and seedlings. Seeds were cultured in either complete darkness (Dark) or a 16-hr light/8-hr dark photoperiod (Light) for 8 weeks. Histograms represent the mean response of seeds in the particular developmental stage representing 10 replicate plates each with 5 subsamples. Histograms with the same letter within developmental stages in both graphs are not significantly different ($\alpha=0.05$). KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium. See Table 2-3 for stages of development.

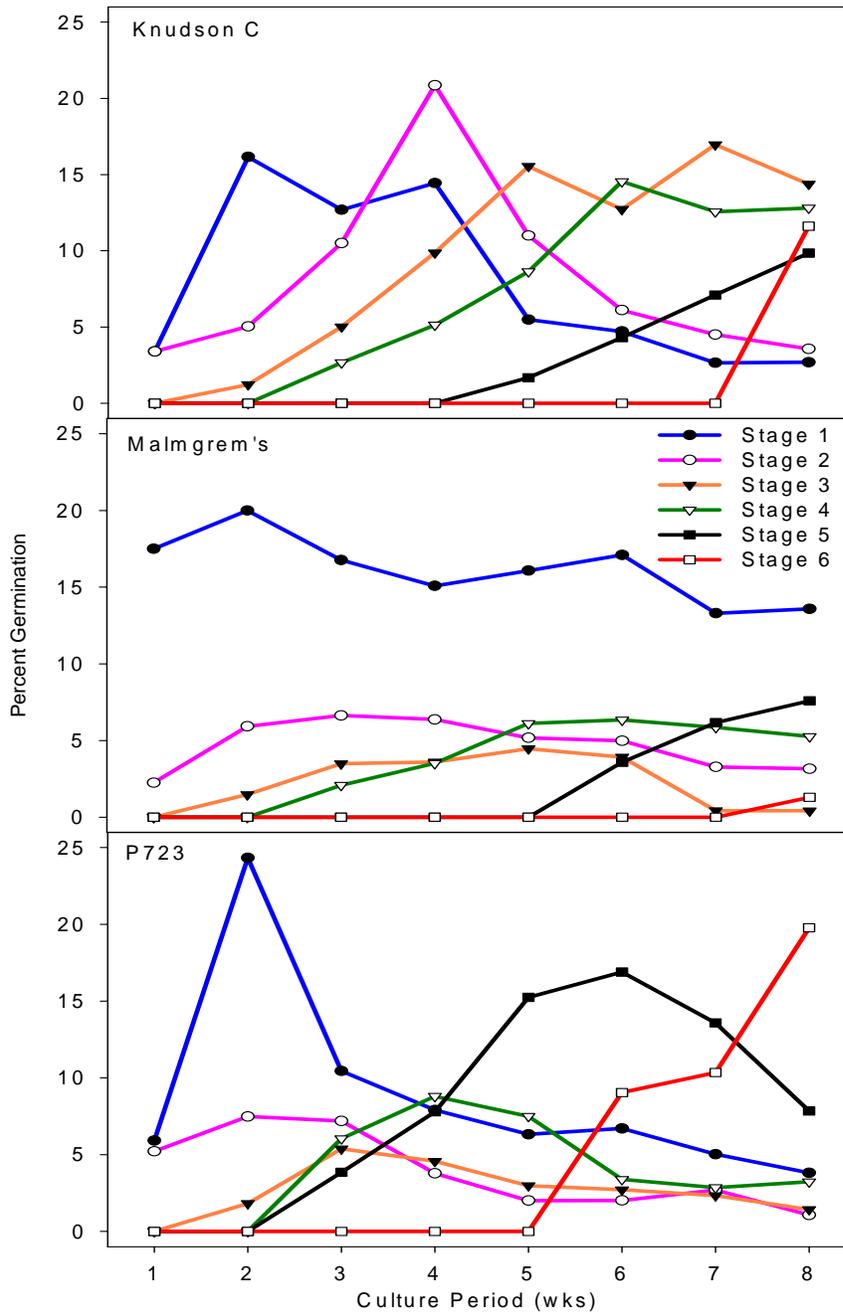


Figure 2-4. Effect of culture media on weekly protocorm and seedling development of *Calopogon tuberosus*. Seeds were cultured in a 16-hour light/8-hour dark photoperiod for 8 weeks. Points on the graphs represent the mean response of ten replicate plates each with five subsamples. See Table 2-3 for stages of development.

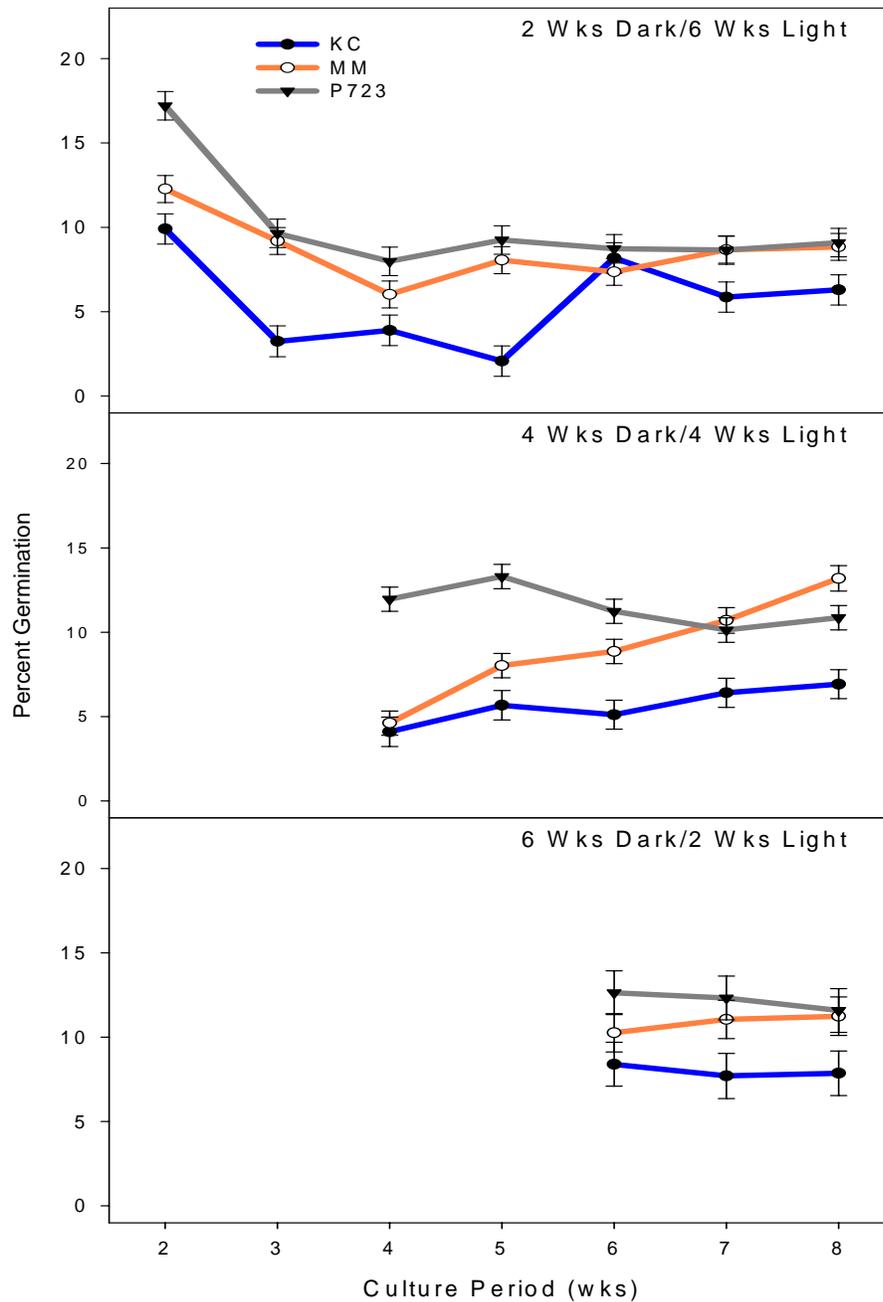


Figure 2-5. Effects of culture media and dark pretreatment on weekly seed germination of *Calopogon tuberosus*. Seeds were cultured for either 2 weeks dark followed by 6 weeks in a 16-hour light/8-hour dark photoperiod, 4 weeks dark followed by 4 weeks in a 16-hour light/8-hour dark photoperiod, or 6 weeks dark followed by 2 weeks in a 16-hour light/8-hour dark photoperiod. Points on the graphs represent the mean response (\pm S.E.) of 10 replicate plates each with 5 subsamples. KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium

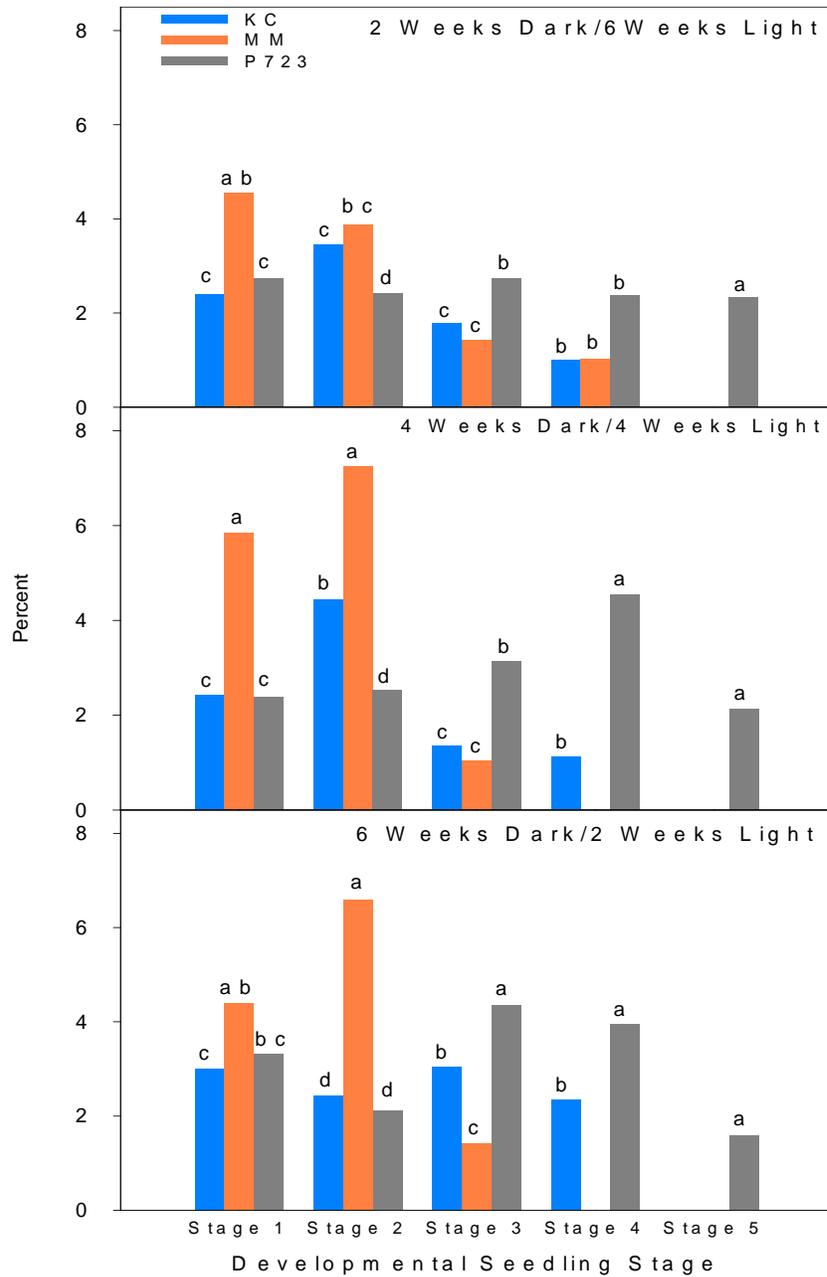


Figure 2-6. Effect of media and dark pretreatment on seedling development after 8 weeks culture. Cultures were incubated for either 2 weeks dark followed by 6 weeks in a 16-hour light/8-hour dark photoperiod, 4 weeks dark followed by 4 weeks in a 16-hour light/8-hour dark photoperiod, or 6 weeks dark followed by 2 weeks in a 16-hour light/8-hour dark photoperiod. Each histogram represents the mean response of 10 replicate plates with 5 subsamples each. Histograms with the same letter in each stage in the three graphs are not significantly different ($\alpha=0.05$) KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium

Qualitative Assessment of Media and Light Interactions on Seed Germination and Seedling Development

Within one week seeds became swollen and white to light green in color. Stage 1 embryos were characterized by an intact or loosened testa (Figure 2-7A). After embryos developed to Stage 1, they quickly progressed in 2-4 days to Stage 2. At this point protocorms were dark green, and the testa was completely detached (Figure 2-7B). During Stage 3, defined shoot apices and rhizoids formed (Figure 2-7C). Following the shoot apex development, the first leaves developed rapidly (Figure 2-7D). During Stage 4, leaf and rhizoid production increased in both length and number (Figure 2-7D). Stage 5 seedlings were characterized by having two fully expanded leaves and the first true root (Figure 2-7E). Stage 6 seedlings normally had one or more true roots as well as two or more leaves (Figure 2-7F).

Seedling development was significantly different among media and light treatments. Seedlings cultured on KC in 8-week 16-hr photoperiod developed to Stage 6 and produced numerous rhizoids, which interlocked causing seedlings to grow closely together (Figure 2-8A). Embryos germinated on KC in complete darkness did not become chlorophyllous, but rather shoot apices were elongated and white (Figure 2-8B). Embryos germinated on MM were the least developed in comparison to those cultured on KC and P723. Embryos germinated on MM in 8 weeks dark developed to Stages 5-6 while embryos cultured in 8-week 16-hr photoperiod did not. Protocorms cultured in 8 weeks dark on MM were etiolated with numerous rhizoids, a characteristic of Stages 3 and 4. A wide range of development from Stages 1-5 was seen in protocorms cultured in the 8-week 16-hr photoperiod on MM (Figure 2-8C and D). The most advanced seedlings were cultured on P723 in the 8-week 16-hr photoperiod. The majority of

embryos germinated on P723 developed to Stage 6 (Figure 2-8E). Embryos cultured on P723 in 8 weeks dark were etiolated with numerous rhizoids (Figure 2-8F).

Seed germination was lower among the dark pretreatments in comparison to seeds germinated in complete darkness or illumination. Among the three dark pretreatments seedling development was similar (Figure 2-9). After 8 weeks, seedlings cultured for 6 weeks in darkness appeared etiolated compared to seeds cultured for 2 weeks dark and 4 weeks dark (Figure 2-9).

Seedling Development

After 12 weeks seedlings cultured on P723 in the 8-week 16-hr photoperiod had longer leaves than seedlings on KC or MM (Figure 2-10). After cultures were removed from complete darkness and moved into light ($91.6 \mu\text{mol m}^{-2} \text{s}^{-1}$), the shoot apices and leaf tips turned light green (Figure 2-10). Seeds cultured on MM in 2 weeks dark/6 weeks light, 4 weeks dark/4 weeks light, and 6 weeks dark/2 weeks light could only be observed under a microscope (Figure 2-10). Seedlings cultured on P723 in 8-week 16-hr photoperiod filled the entire culture plate and leaves reached the top of the petri plate. After 12 weeks culture small corms began to form at the base of seedlings cultured on P723.

After 16 weeks culture seedlings from all treatments were transferred to *Phytotechnology Culture Boxes* for further growth and development (Figure 2-16). Corms several millimeters in diameter readily formed on seedlings cultured on P723. Corm formation occurred regardless of initial light treatment. After 24 weeks growth (8 weeks in Culture Boxes) seedlings cultured initially in 8-weeks light and 8-weeks dark were evaluated for growth and development (Figure 2-15). Corms developed independently of plant size after 7 months culture (Figure 2-17).

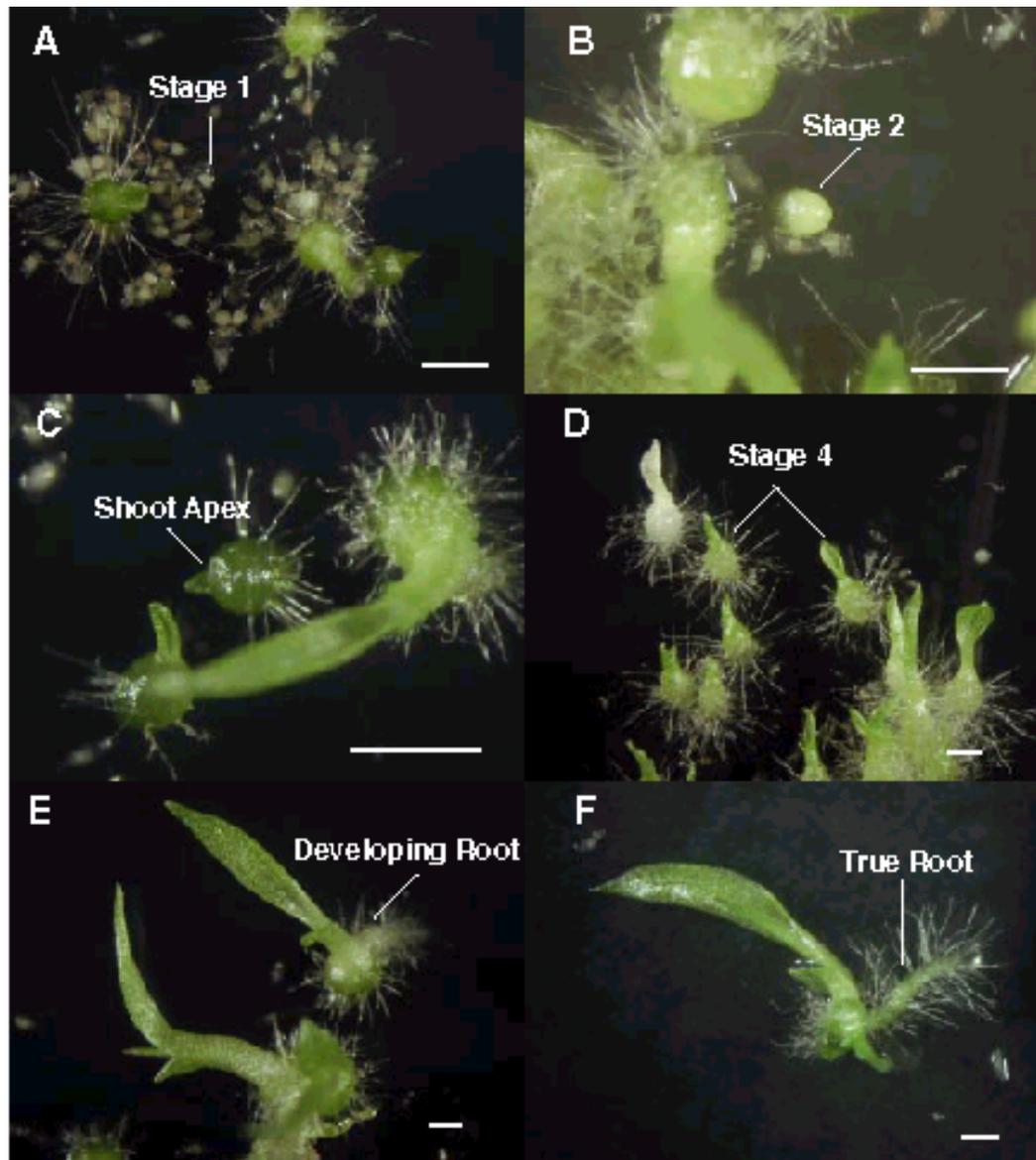


Figure 2-7. Stages of *in vitro* seed germination and seedling development of *Calopogon tuberosus*. A) Stage 1 imbibed embryo. B) Stage 2 protocorm with detached testa. C) Stage 3 protocorm with pointed shoot apex. D) Stage 4 protocorm with developing leaves and rhizoids. E) Stage 5 seedling with developing root, 1 fully expanded leaf, 1 developing leaf, and swollen shoot base. F) Stage 6 seedling with true root, two leaves, and swollen shoot base. Scale bars=1 mm

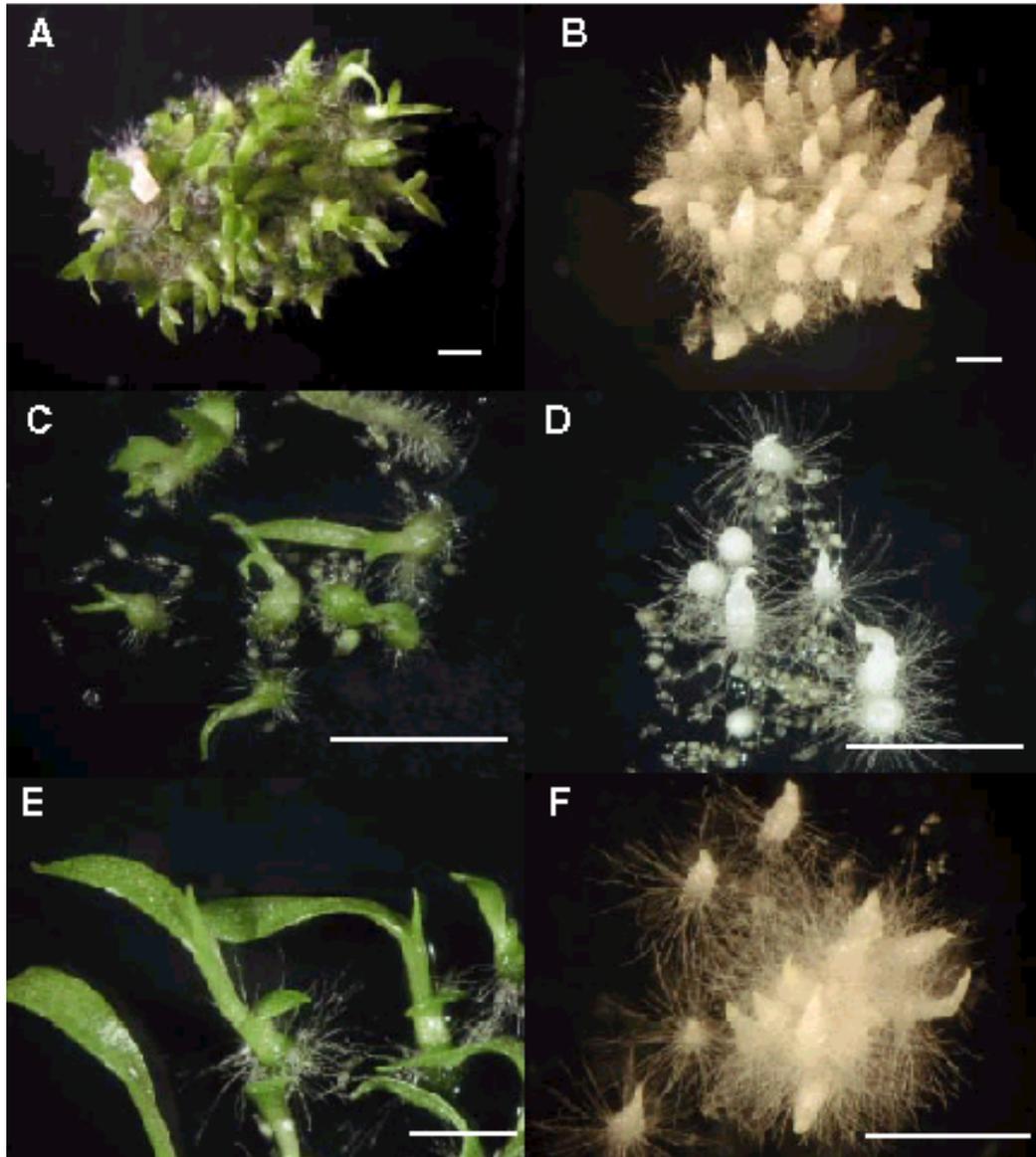


Figure 2-8. Comparative effects of media and light on protocorm and seedling development of *Calopogon tuberosus* after 8 weeks culture. A) Seeds cultured on KC (8-week 16-hr photoperiod). B) Seeds cultured on KC (8 weeks dark). C) Seeds cultured on MM (8-week 16-hr photoperiod). D) Seeds cultured on MM (8 weeks dark). E) Seeds cultured on P723 (8-week 16-hr photoperiod). F) Seeds cultured on P723 (8 weeks dark). KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium Scale bars= 5 mm

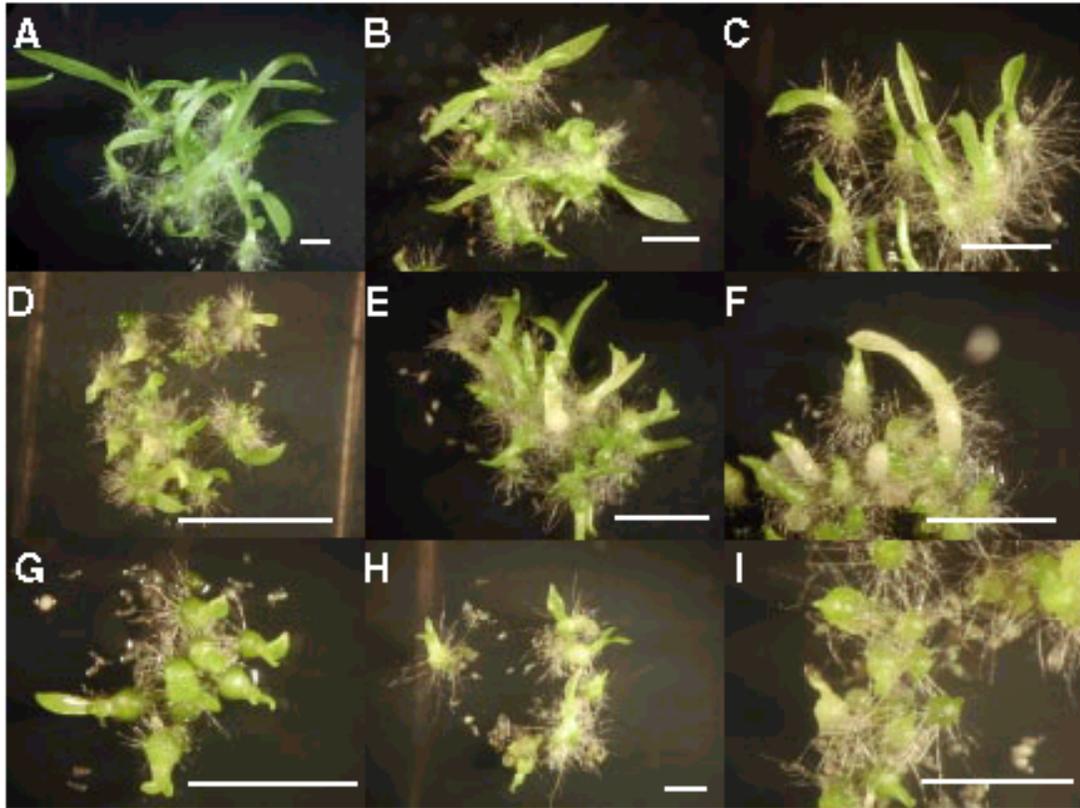


Figure 2-9. Seed germination and seedling development of *Calopogon tuberosus* after 8 weeks culture. From left to right: 2 weeks dark/6 weeks light incubation, 4 weeks dark/4 weeks dark incubation, 6 weeks dark/2 weeks light incubation. A-C) Seeds cultured on P723. D-F) Seeds cultured on KC. G-I) Seeds cultured on MM. KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium Scale bars=5 mm

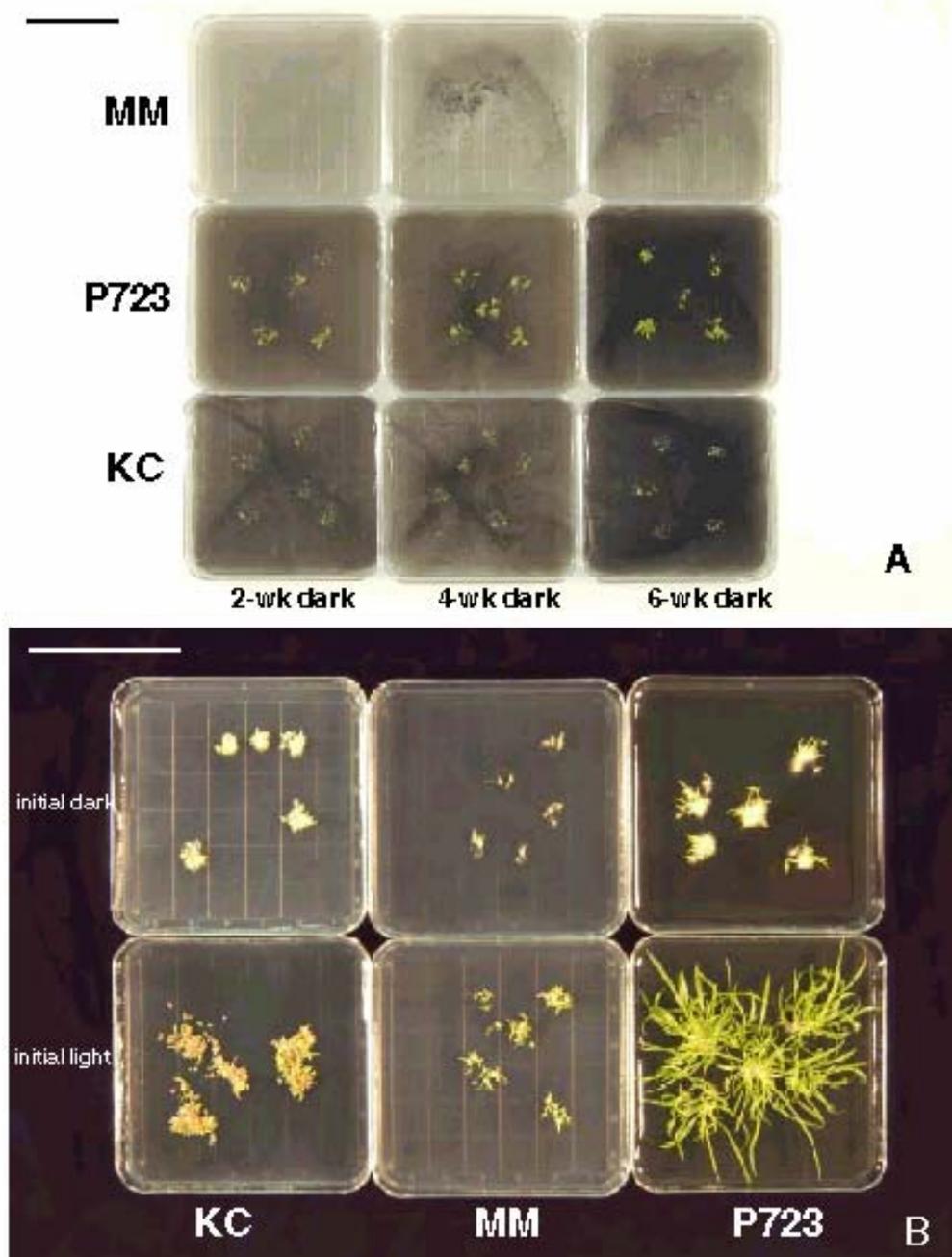


Figure 2-10. Comparative effect of media and light treatment on seed development of *Calopogon tuberosus* after 12 weeks culture. After 8 weeks cultures were placed under a 16-hour light/8-hour dark photoperiod. A) 12 week cultures initially incubated in the 2 weeks dark/6 weeks light incubation, 4 weeks dark/4 weeks dark incubation, 6 weeks dark/2 weeks light incubation. B) 12 week cultures initially incubated for 8 weeks in complete darkness and the 16-hour light/8-hour dark photoperiod. KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium
Scale bars=5 cm

Seedlings cultured on P723 had longer leaves and roots than seedlings cultured on MM and KC (Figure 2-11). Seedlings cultured on P723 in the initial 8-week 16-hr photoperiod had significantly longer leaves than seedlings cultured on P723 in the 8-week dark period. Root length was significantly lower on seedlings cultured on KC and MM. Initial light had a significant effect on leaf number for seedlings cultured on KC and MM (Figure 2-12). The lowest leaf production occurred on KC in the initial 8-week dark period. Seedlings cultured on P723 produced the highest number of roots (Figure 2-12).

Fresh and dry weights were significant among culture media (Figure 2-13 and Figure 2-14). Seedlings cultured on P723 in the initial 8-week light period had significantly greater fresh weight compared to seedlings cultured on KC, MM, and P723 in the 8-week dark period. Seedlings on MM in the initial 8-week light period had a higher dry weight than those in the initial 8-week dark period. Dry weight was higher for seedlings cultured on P723 in the initial 8-week dark period than those cultured in the 8-week light period.

Observations on Cold-Storage

After 7 months culture, the majority of leaves turned brown, but corms remained green (Figure 2-18). Not all seedlings exhibited shoot die-back. Whitlow (1996) found that placing seedlings in cold-storage set them on a synchronized growth cycle. In this study, seedlings not placed in cold-storage ($91.6 \mu\text{mol m}^{-2} \text{s}^{-1}$ at $25 \pm 2 \text{ C}$) had variable growth rates with some beginning new growth (Figure 2-18). Seedlings were placed under a 16-hr photoperiod at $91.6 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $25 \pm 2 \text{ C}$ after cold-storage. Corms remained green throughout cold-storage in complete darkness. Seedlings in cold-storage

(10.9 ± 0.7 C) for 1 month started new growth one week after removal from cold-storage. Many seedlings in 1 month cold-storage rapidly grew new shoots within 2 weeks after cold-storage. Seedlings placed in cold-storage for 2 months grew slowly after being placed under room condition as evident by the small shoots.

Acclimatization

Seedlings of *C. tuberosus* acclimated to greenhouse conditions. Original shoots on the majority of corms showed signs of browning after 5 weeks and completely senesced after 7 weeks (Figure 2-23E and F). Among all treatments 53% of seedlings regenerated new growth within the 12-week acclimatization. New shoots regenerated from original corms after 8 weeks under greenhouse conditions, and new corms formed on several seedlings (Figure 2-23E and F). Seedling survivorship was high in all potting media. Survival was classified by the presence of a nonactive or actively growing corm. Survivorship on sphagnum, vermiculite, peat/sand, and Fafard mix was 89% (64 of 72), 90% (65 of 72), 90% (65 of 72), and 88% (63 of 72), respectively. Drainage was greatest in vermiculite and slowest in the Fafard mix.

Seedling leaf production was not significantly different among potting media (Figure 2-19). Seedling root production in sphagnum was significantly lower than seedlings in vermiculite, peat/sand, and Fafard mix (Figure 2-19). Seedling leaf and root lengths were similar for all potting media (Figure 2-20). Seedling fresh weight, dry weight (Figure 2-21), and corm diameter (Figure 2-22) were significantly lower in vermiculite compared to seedlings in sphagnum, peat/sand, and Fafard mix.

Discussion

Media Comparison

Successful *in vitro* seed germination has been previously reported for *Calopogon tuberosus*. Arditti, et al. (1985) reported near 100% germination in two months and 5-10 cm long seedlings in four months. Henrich et al. (1981) reported 25% germination in 29 days. A tetrazolium test yielded 35% viable seed for *C. tuberosus* (S. Stewart, unpublished data). In the present study, maximum germination rates of all seeds ranged from a low of 5% to a high of 50% after 8 weeks culture. If including just viable seed, germination rates ranged from 14% to 100%. Unlike previous reports that seed germination was similar on variety of culture media (Whitlow, 1996; Whitlow, pers. comm.), seed germination of *C. tuberosus* is significantly affected by culture media type. This may be a result in differences in plant genotypic or ecotypic requirements for germination.

A major difference between KC, MM, and P723 is the form and concentration of nitrogen (Table 2-2 and Table 2-3). While KC and P723 contain inorganic nitrogen sources (ammonium and nitrate), MM, as prepared by *Phytotechnology Laboratories*, contains an organic nitrogen source in the form of the amino acid glycine. Malmgren (1992, 1996) and Van Waes and Debergh (1986b) found that germination rates of several cold-hardy terrestrial orchids were higher on media containing organic nitrogen. Malmgren suggested that an organic nitrogen source provided in the form of an amino acid might be more readily available than an inorganic nitrogen source due to simplified forms of nitrogen.

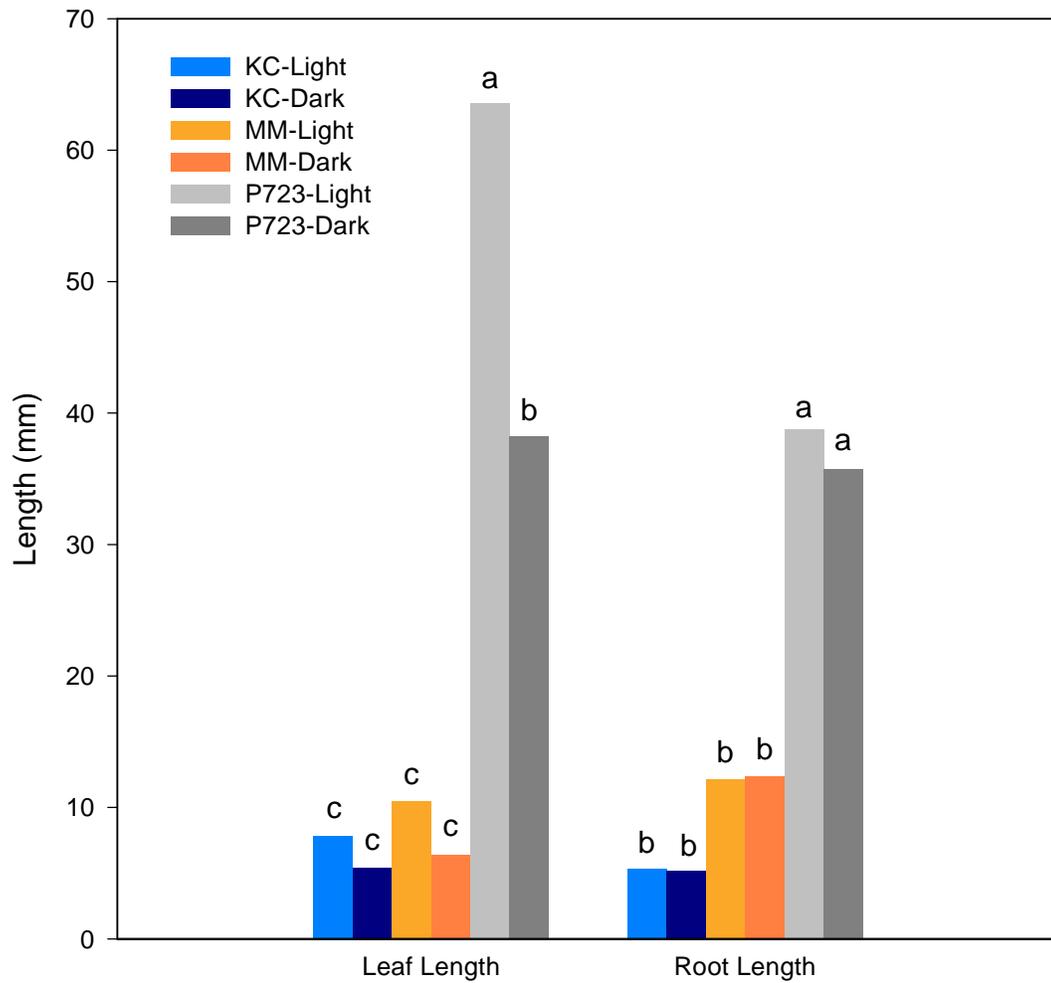


Figure 2-11. Comparative effect of media and light treatment on leaf and root length of *Calopogon tuberosus* seedlings after 24 weeks culture. Histograms represent the mean response of 50 randomly selected seedlings. Histograms with the same letter are not significantly different ($\alpha=0.05$). KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium; Light: initial 8 week culture under a 16-hour light/8-hour dark photoperiod; Dark: initial 8 week culture under complete darkness.

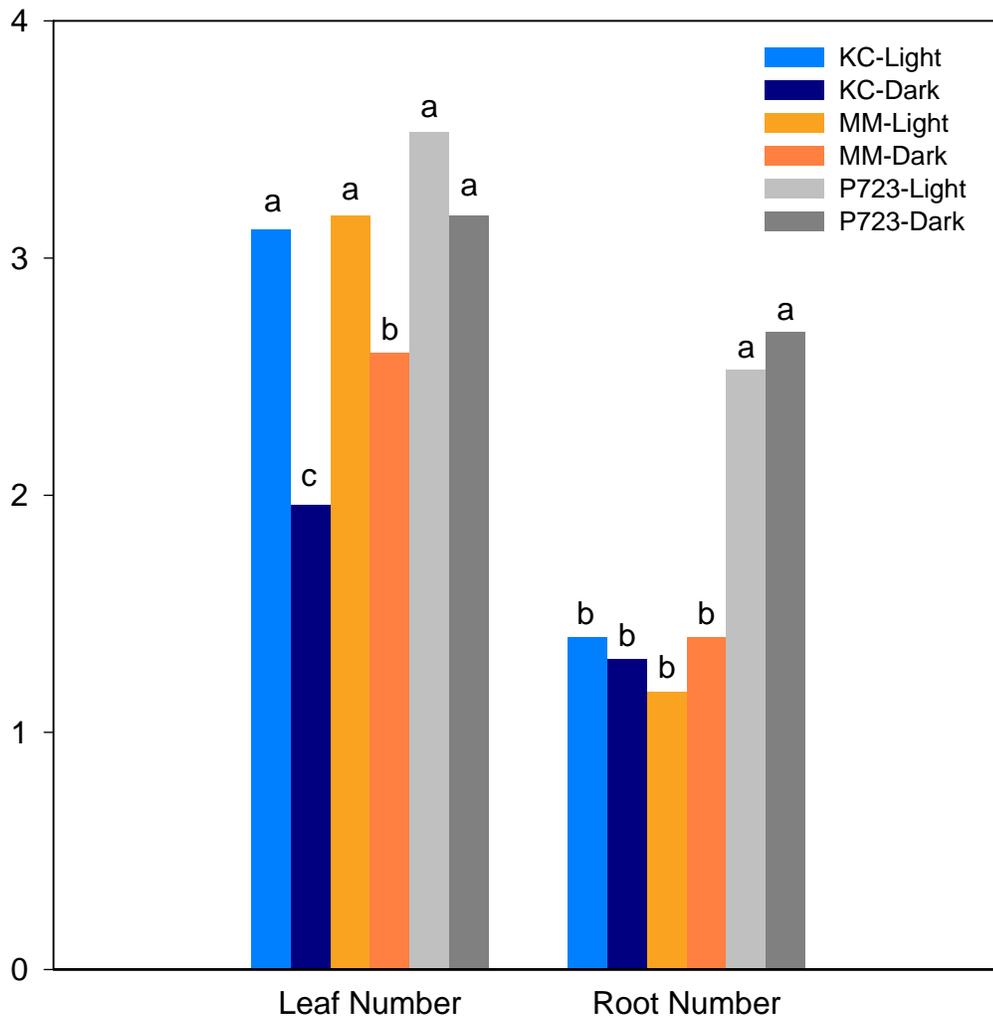


Figure 2-12. Comparative effect of culture media and light treatment on leaf and root number of *Calopogon tuberosus* seedlings after 24 weeks culture. Histograms represent the mean response of 50 randomly selected seedlings. Histograms with the same letter are not significantly different ($\alpha=0.05$). KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium; Light: initial 8 week culture under a 16-hour light/8-hour dark photoperiod; Dark: initial 8 week culture under complete darkness.

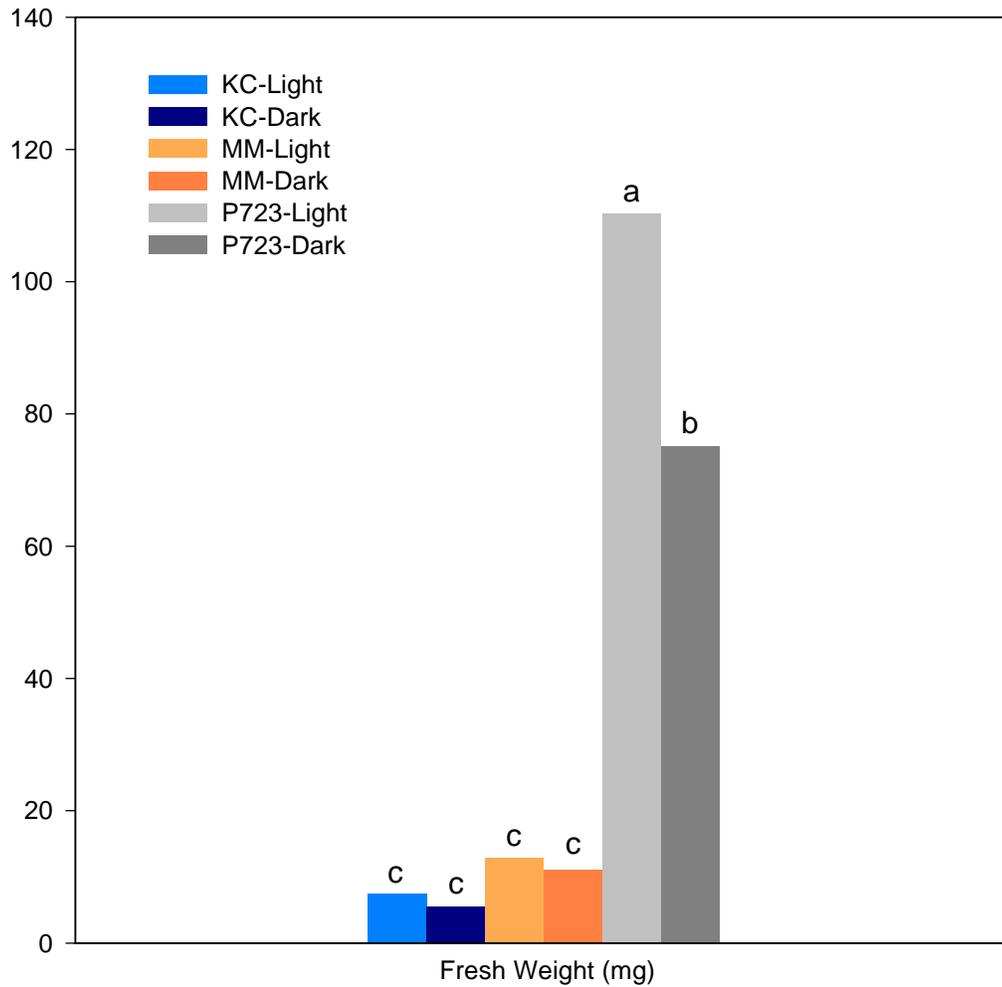


Figure 2-13. Comparative effect of culture media and light treatment on fresh weight of *Calopogon tuberosus* seedlings after 24 weeks culture. Histograms represent the mean response of 50 randomly selected seedlings. Histograms with the same letter are not significantly different ($\alpha=0.05$). KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium; Light: initial 8 week culture under a 16-hour light/8-hour dark photoperiod; Dark: initial 8 week culture under complete darkness.

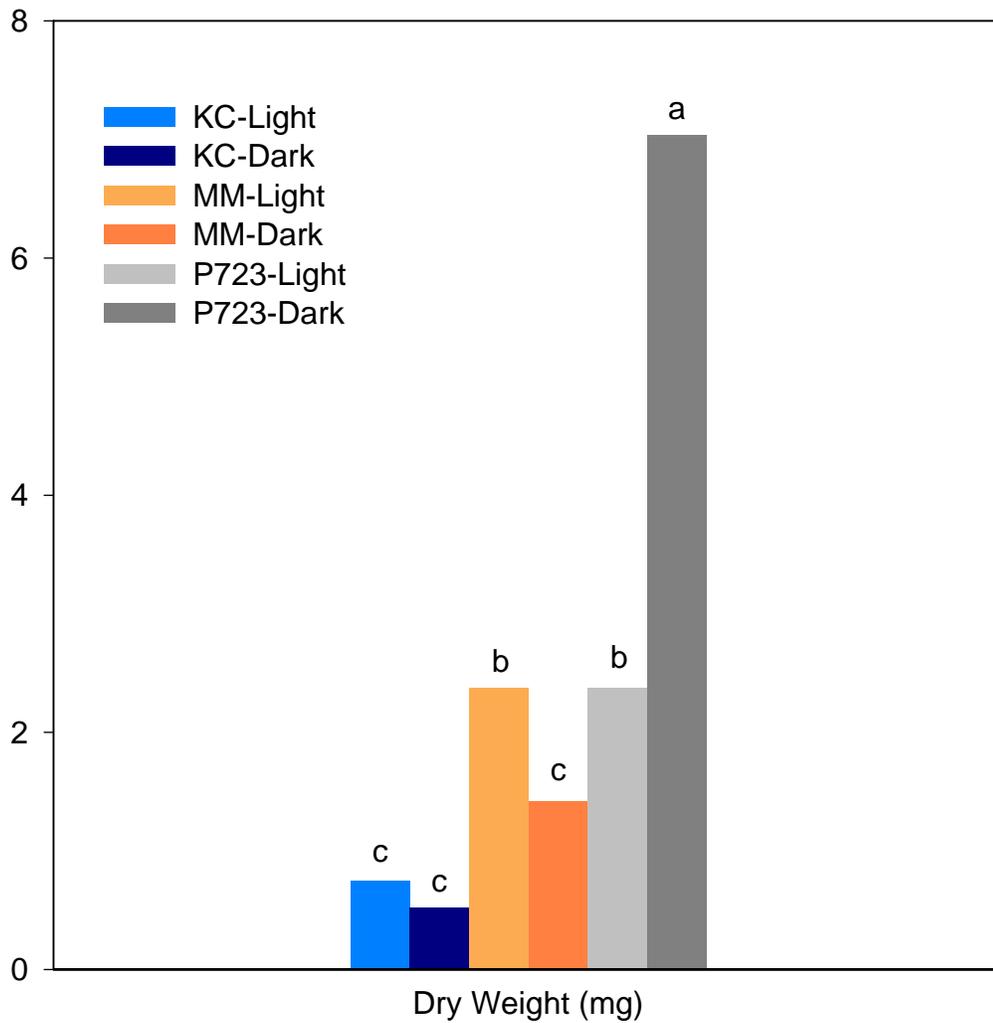


Figure 2-14. Comparative effect of culture media and light treatment on dry weight of *Calopogon tuberosus* seedlings after 24 weeks culture. Histograms represent the mean response of 50 randomly selected seedlings. Histograms with the same letter are not significantly different ($\alpha=0.05$). KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium; Light: initial 8 week culture under a 16-hour light/8-hour dark photoperiod; Dark: initial 8 week culture under complete darkness.

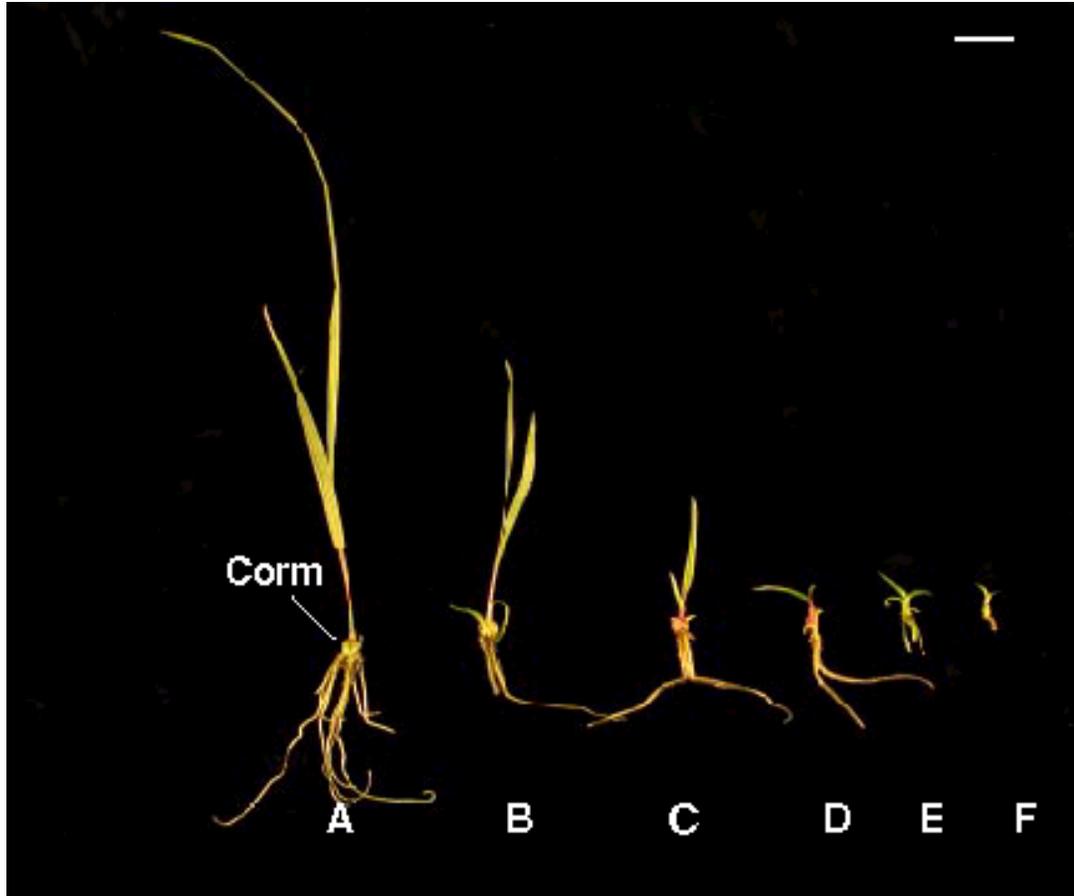


Figure 2-15. Comparative seedling development of *Calopogon tuberosus* after 24 weeks culture. A) Seedling with developing corm cultured on P723 and initially incubated in a 16-hour light/8-hour dark photoperiod. B) Seedling with developing corm cultured on P723 and initially incubated in complete darkness. C) Seedling with developing corm cultured on KC and initially incubated in a 16-hour light/8-hour dark photoperiod. D) Seedling cultured on KC and initially incubated in complete darkness. E) Seedling cultured on MM and initially incubated in a 16-hour light/8-hour dark photoperiod. F) Seedling cultured on MM and initially incubated in complete darkness. Scale bar=1 cm



Figure 2-16. Seedling development of *Calopogon tuberosus* in Phytotech Culture Box after 16 weeks culture. Scale bar=15mm

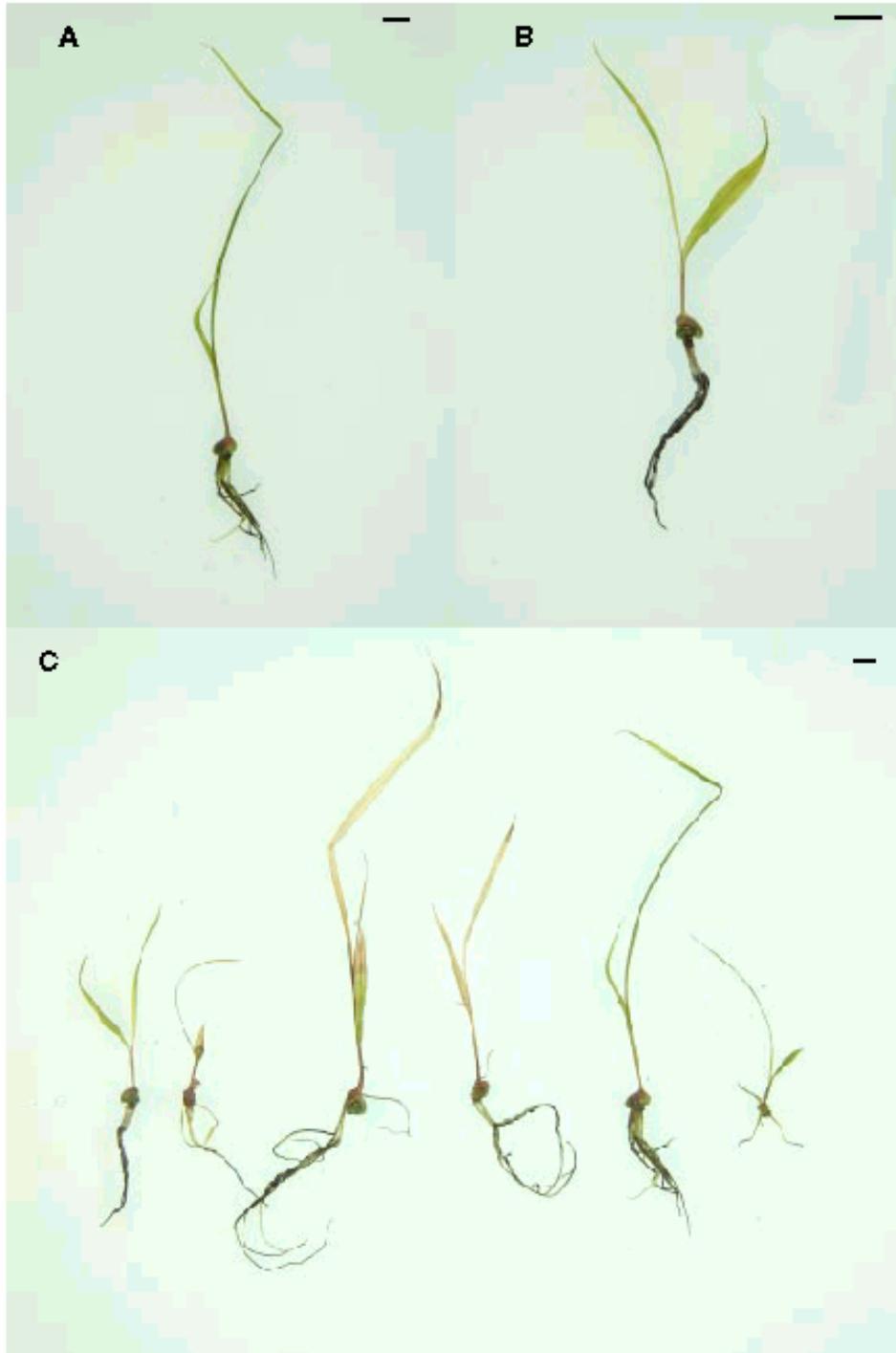


Figure 2-17. Corm development after 7 months *in vitro* culture of *Calopogon tuberosus* seedlings. A) Large seedling. B) Small seedling. C) Various sizes of seedlings with developing corms. Scale bars=1 cm

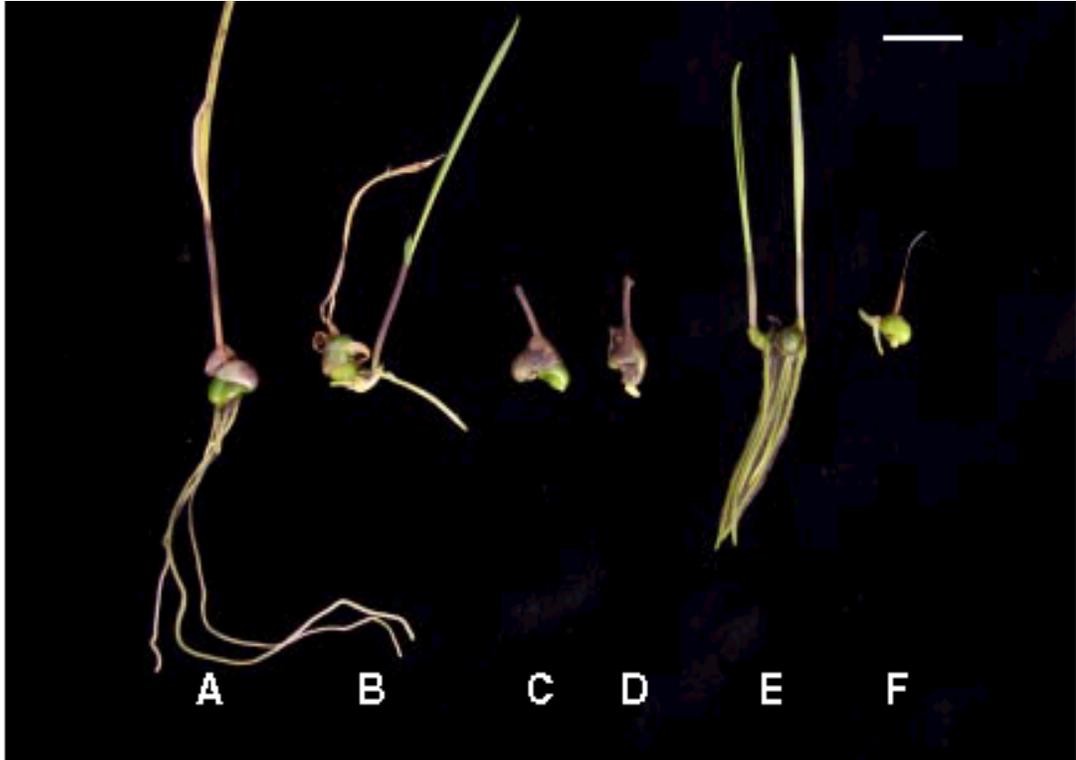


Figure 2-18. Effects of cold-storage on seedlings of *Calopogon tuberosus*. Picture taken 3 weeks after removal from cold-storage at 10.9 ± 0.7 C. A) Seedling with no cold-storage and evident shoot die-back. B) Seedling with no cold-storage. A new shoot is growing from the original corm. C) Seedling with 2 months cold-storage and no evident new growth. D) Seedling with 2 months cold-storage with new small growth. E) Seedling with 1 month cold-storage and 2 new shoots. F) Seedling with 1 month cold-storage and small new growth. Scale bar=1 cm

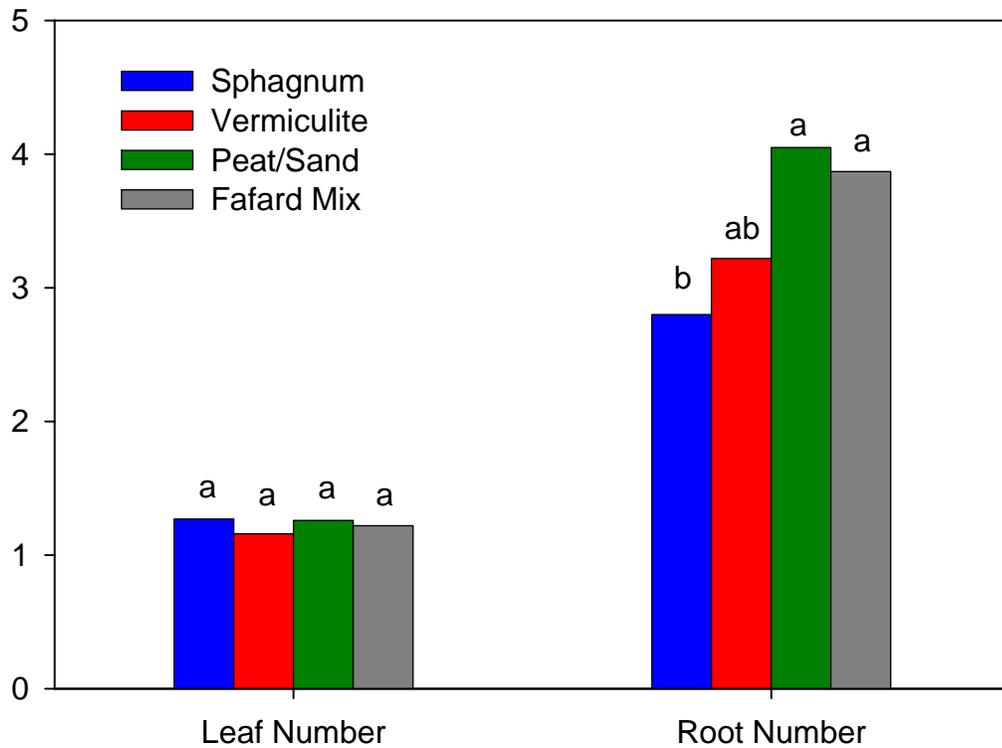


Figure 2-19. Potting media effects on leaf and root production of *Calopogon tuberosus* seedlings acclimatized to greenhouse conditions for 12 weeks. Histograms represent the mean response of 8 replicate-trays (blocks). Each block contained the four potting media with 9 seedlings per potting medium. Histograms with the same letter are not significantly different ($\alpha=0.05$).

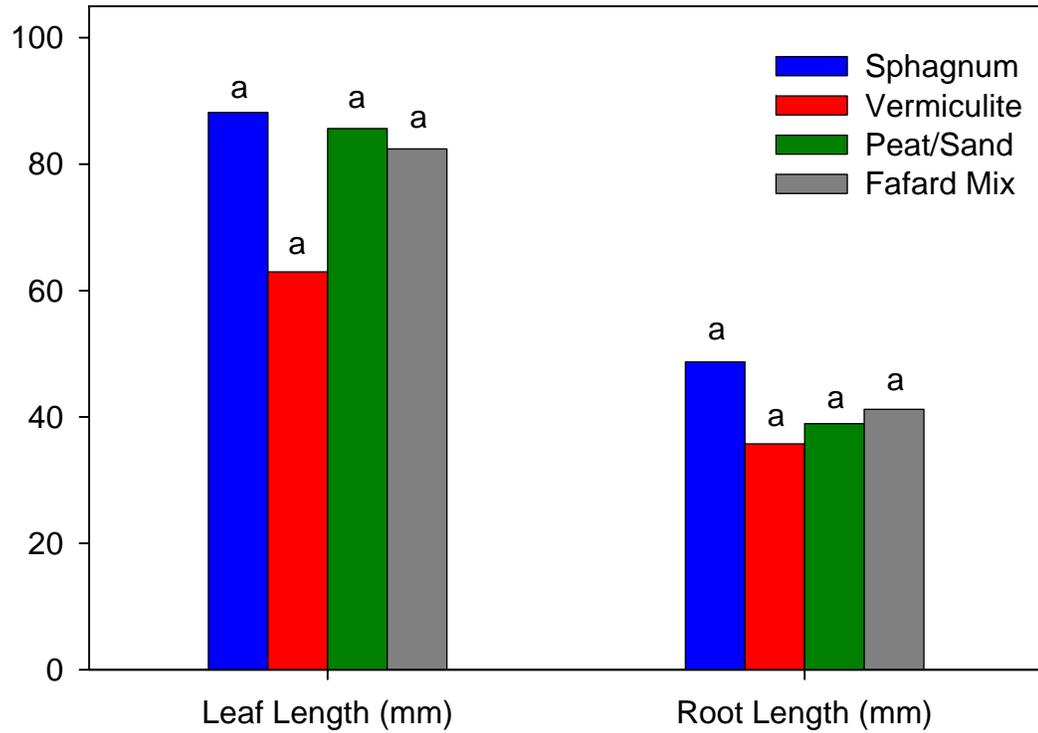


Figure 2-20. Potting media effects on leaf and root length of *Calopogon tuberosus* seedlings acclimatized to greenhouse conditions for 12 weeks. Histograms represent the mean response of 8 replicate-trays (blocks). Each block contained the four potting media with 9 seedlings per potting medium. Histograms with the same letter are not significantly different ($\alpha=0.05$).

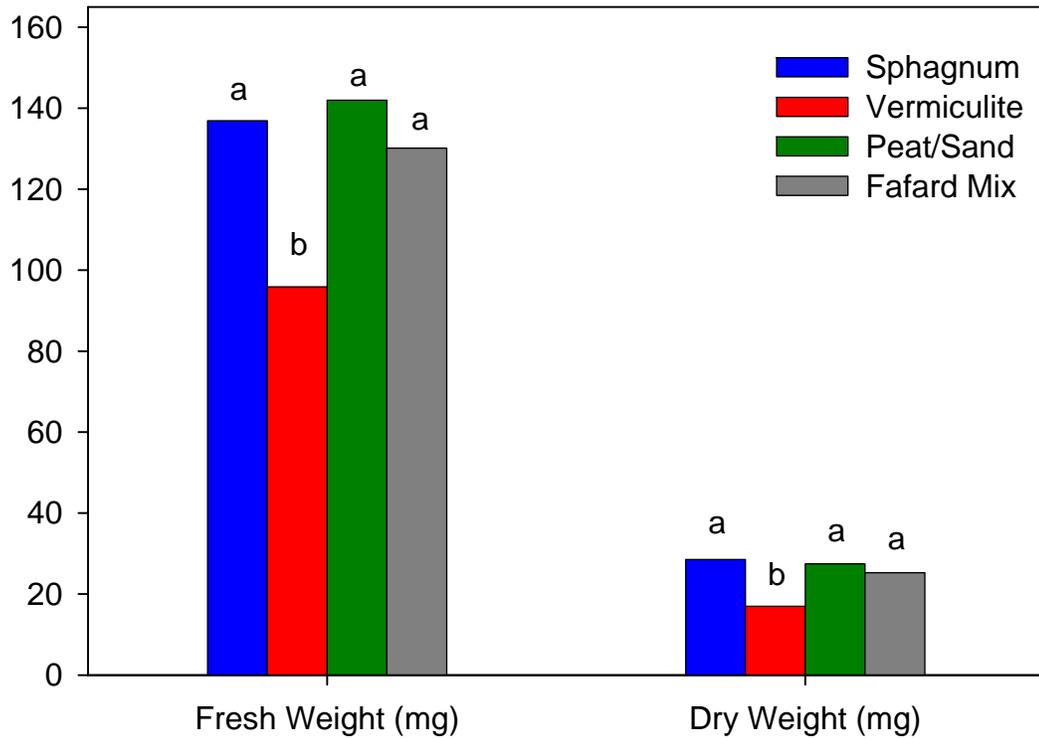


Figure 2-21. Potting media effects on fresh and dry of *Calopogon tuberosus* seedlings acclimatized to greenhouse conditions for 12 weeks. Seedlings were placed in a drying oven at 60 C for 48 hours to measure dry weight. Histograms represent the mean response of 8 replicate-trays (blocks). Each block contained the four potting media with 9 seedlings per potting medium. Histograms with the same letter are not significantly different ($\alpha=0.05$).

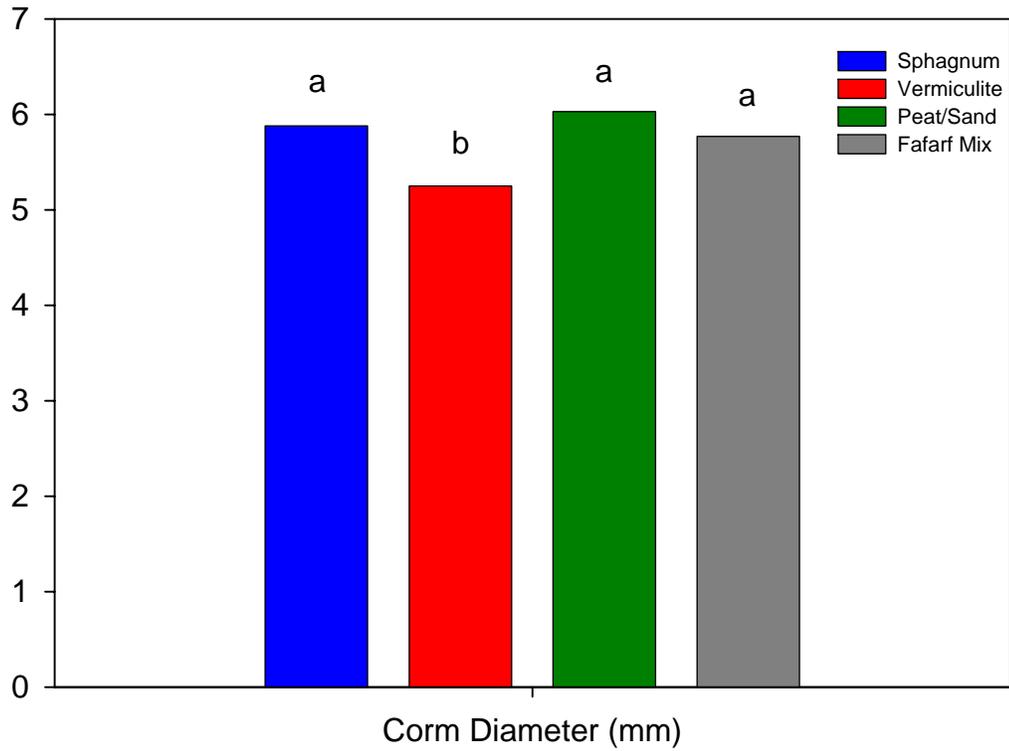


Figure 2-22. Potting media effects on corm diameter of *Calopogon tuberosus* seedlings acclimatized to greenhouse conditions for 12 weeks. Corms were measured from the two furthest points. Histograms represent the mean response of 8 replicate-trays (blocks). Each block contained the four potting media with 9 seedlings per potting medium. Histograms with the same letter are not significantly different ($\alpha=0.05$).

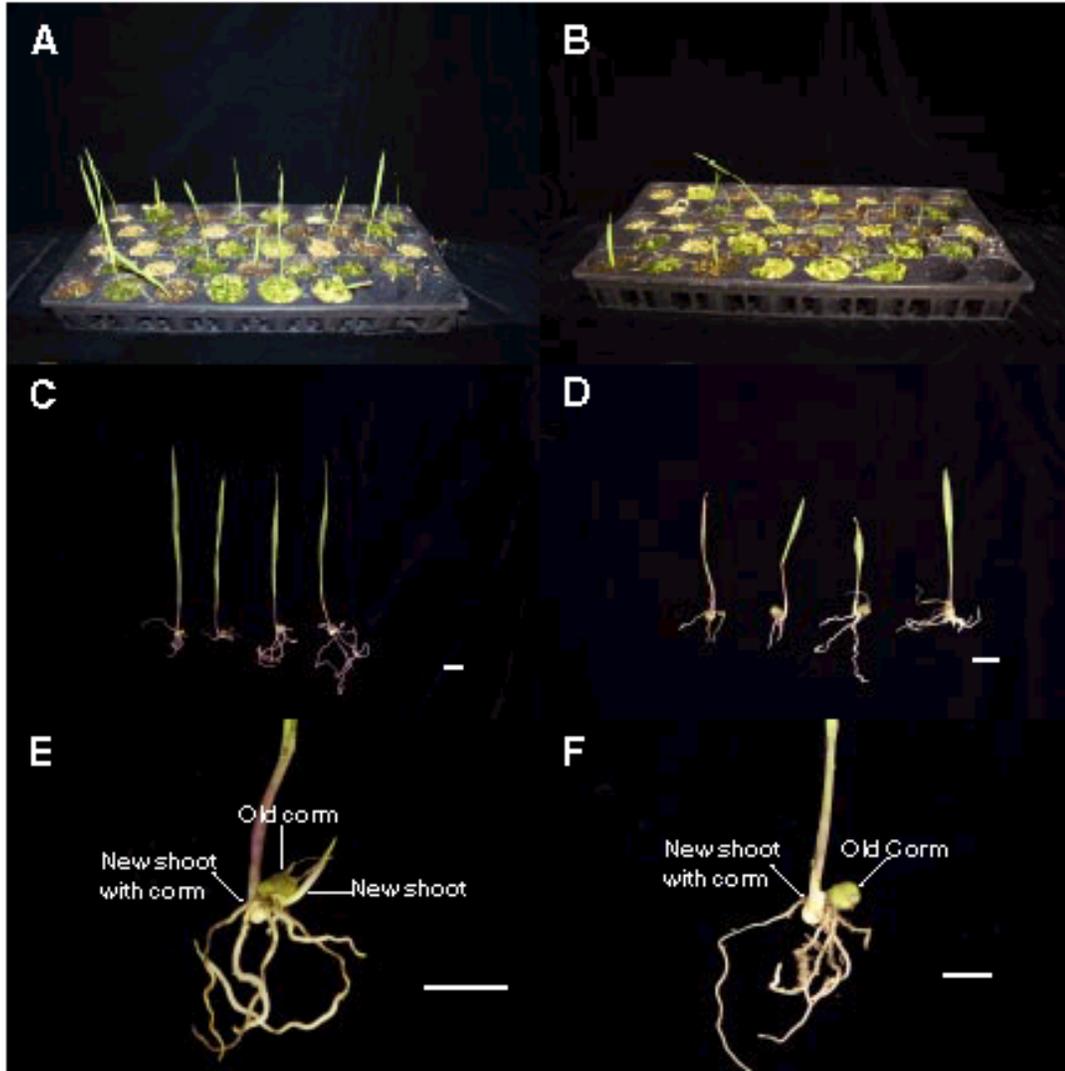


Figure 2-23. Twelve-week greenhouse acclimatization of *Calopogon tuberosus* seedlings. A) Block replication of four potting media with rapid growth and development of seedlings. B) Block replication of four potting media with slow growth and development of seedlings. C) Large-sized seedlings grown in four potting media. From left to right: 1:1 peat/sand; vermiculite; Fafard mix; sphagnum moss. D) Average-sized seedlings grown in four potting media. From left to right: 1:1 peat/sand; vermiculite; Fafard mix; sphagnum moss. E) Old corm with 2 new shoots. A new shoot and corm is forming to the left while a young shoot is forming on the right side of the corm. D) Old corm with new shoot/corm. Scale bars=1 cm

Raghavan (1964) found that only certain amino acids are as effective as ammonium in germinating *Cattleya* seeds. Glycine, the simplest amino acid, decreased overall germination of *Cattleya* seeds from 53% to 41% when compared to ammonium nitrate. However, seed germination with other amino acids, such as arginine, proline, and glutamine, was similar to germination with ammonium nitrate (Raghavan, 1964). Spoerl and Curtis (1948) obtained similar results. Glycine significantly reduced germination of *Cattleya* seeds over 2 months while germination with arginine was similar compared to ammonium nitrate. However, within 5 months germination on the glycine containing medium increased from 22.5% to 64% and from 64% to 78% within 7 months. Over the 8-week culture period in the present study, seed germination on MM in 8-weeks light progressively increased compared to germination on KC and P723. Spoerl and Curtis (1948) suggested that amino acid enzyme systems within developing embryos change eventually. Amino acids may not be available as nitrogen sources initially, but are metabolized within time. Raghavan (1964) and Spoerl and Curtis (1948) mentioned that embryos from various orchid species respond differently to various amino acids during germination, and therefore further investigation should be carried out. Since not all amino acids are beneficial for seed germination, combinations of amino acids may increase germination (Spoerl and Curtis, 1948).

Ziegler et al. (1967) studied the effects of Edamin, a lactalbumin hydrolysate with peptides and 18 amino acids, on the germination of a *Cattleya x Laelia* hybrid. On the media with Edamin, embryos became greener faster than those on media without Edamin. Dry weight of seedlings was higher and seedlings were more uniform in the presence of Edamin. Tissue analysis of seedlings cultured on Edamin yielded increased levels of

amino acids. Interestingly, glutamine, asparagine, and gamma amino butyric acid were detected in seedling tissue, but these amino acids were not found in Edamin. The researchers suggested that Edamin was used as an amino acid building component.

Raghavan and Torrey (1964) and Curtis (1947) found that seeds of a *Cattleya* hybrid germinated more efficiently on media containing an ammonium source rather than a nitrate or nitrite source. Knudson C and P723 contain both ammonium and nitrate. The initial high germination rates (Figure 2-2) in the first several weeks on KC and P723 may be due to the presence of ammonium in the media. Ammonium content was highest in KC, and may contribute to the high overall germination rates. Curtis and Spoerl (1948) found that a high ratio of ammonium to nitrate (4.2:1) was beneficial for germination of *Cattleya* seedlings. The ammonium to nitrate ratio in P723 is 0.5:1, while the ratio in KC is 1.12:1 (Table 2-2). This difference may have promoted the increased germination on KC. Raghavan and Torrey (1964) found that nitrate reductase activity was evident after 60 days culture, and therefore nitrate uptake was unavailable until that point. P723 contained slightly more nitrate than KC, and this might have promoted the excelled growth of seedlings cultured on P723.

P723 also contains 0.2% peptone, an additional source of nitrogen (Table 2-1). The exact amount of nitrogen contributed by peptone is uncertain. Curtis (1947) found that the addition 0.05% peptone to culture media significantly increased seed germination of *Paphiopedilum* and *Vanda* species. Seed germination of *Habenaria clavellata*, a native orchid of the United States, was reduced in the presence of peptone. Peptone was also responsible for increased uniformity of seedling development. The presence of peptone in P723 also may be responsible for increased seedling development.

Seed germination of *C. tuberosus* was higher on KC than on MM; however, germination rates were not significantly different between MM and P723 (Figure 2-1). Seedling growth and development was higher on P723 in comparison to MM and KC (Figure 2-4). Although P723 did not have the highest total ionic salt content, it did contain higher micronutrient concentrations than either MM or KC (Table 2-2). Gamborg et al. (1976) reported that micronutrients such as boron, manganese, zinc, molybdenum, copper, cobalt, and iron are required in plant tissue culture media. All these micronutrients are found in P723 while only iron and manganese are found in KC and MM.

Gamborg et al. (1976) stated that molybdenum should be included in any plant culture medium. Although required in small quantities, molybdenum is essential for nitrogen assimilation, biosynthesis of abscisic acid, purine catabolism, and sulfur reduction (Mendel and Hänsch, 2002). Breddy (1953) found that the addition of molybdenum along with other micronutrients to culture media increased both seed germination and seedling growth. Villora et al. (2002) found that higher ammonium nitrate applications to eggplant significantly decreased the concentration of molybdenum in leaf tissue. The reduced molybdenum concentration was responsible for a decrease in the capacity of the plant to reduce nitrate to ammonium and ammonium to amino acids. The enhanced germination, growth, and development of seeds cultured on P723 may be influenced by the presence of molybdenum. Nitrogen sources may be more efficiently assimilated on P723 causing the seeds to develop to Stage 6 quickly. Reduced seedling development on KC and MM may be influenced by the lack of molybdenum. Due to the

higher concentration of ammonium in KC, any endogenous molybdenum in seedlings may be repressed by the higher ammonium concentration.

Niacin has been found to be beneficial for orchid seed germination (Arditti, 1967b). Niacin and its derivatives are essential for metabolism as components of NAD^+ and NADP^+ . P723 contains nicotinic acid, a derivative of niacin. Metabolism may be more efficient in seeds cultured on P723 than those on MM or KC. This is a possible explanation for the increased growth and vigor seeds observed on P723.

Light Treatment Effects

The roles of light and darkness in seed germination have not been sufficiently examined for *C. tuberosus*. While Whitlow (1996) recommended light incubation, others recommended dark incubation (Henrich et al., 1981; Myers and Ascher, 1982). Results of this study have shown that while dark incubation promotes germination while light incubation is beneficial for rapid seedling development. In contrast, seed germination of many cold-hardy terrestrial orchids is inhibited by light incubation (Arditti et al., 1981; Van Waes and Deberg, 1986b). Many cold-hardy terrestrial orchids inhabit dry, shaded forest floors, where seeds are not exposed to direct sun. Stoutamire (1964) found that species of wet, open or partially shaded locations, such as *C. tuberosus*, germinate quickly and develop rapidly. Species found in open areas are exposed to higher light levels than species found on the dark forest floor, and therefore will germinate in the presence of light (Stoutamire, 1964).

Seeds of *C. tuberosus* incubated under light conditions quickly became green suggesting early onset of photosynthesis. In comparison to light incubation, seeds cultured in complete darkness remained white until placed under light. Seedlings cultured in complete darkness were etiolated under dark conditions due to the lack of

light. Upon transfer of dark incubated cultures to light, protocorms quickly became green. After 24 weeks, growth and development was still suppressed compared to seeds germinated under the initial 8-week 16-hr photoperiod (Figure 2-15). Photosynthates, such as carbon and sugars, may have been accumulated more efficiently during the initial 8-week light period, and therefore carry-over effects of the photosynthates may be responsible for subsequent development.

The negative effects of a dark pretreatment before light on seed germination have been reported (Rasmussen et al., 1990; Zettler and McInnis, 1994). Under natural conditions seeds of terrestrial orchids germinate in soil, and are exposed first to light then darkness. Low germination rates among the dark pretreatments may be due to conditions of dark before light under an environment favorable for germination. Although seeds were stored in light, the storage conditions were not beneficial for germination due to the lack of water and nutrients.

Cold-Storage

Whitlow (1996) reported the beneficial effects of cold-storage on dormant seedlings, and the preliminary in this study confirm these results. After 6 months culture, some seedlings began leaf senescence, while other seedlings continued growth. Although cold-storage was not necessary to break dormancy in all seedlings, seedlings not placed in cold-storage may or may not have formed new shoots. Whitlow (1996) found that seedlings not placed in cold-storage remained dormant indefinitely. The time frame to form new shoots among non-cold treated seedlings can be variable. In order to maintain seedlings on the same time schedule, all seedlings should be placed in cold-storage for some period of time. One month cold-storage was beneficial compared to two months cold storage. Seedlings in one month cold-storage quickly formed new shoots upon

removal. Stoutamire (1964) reported that seedlings needed 4-6 weeks of cold-storage to break dormancy. Whitlow (1996) placed seedlings in cold-storage for 3 months, which was repeated after another 5-6 months of seedling growth in culture. The findings in this study are in accordance with Stoutamire (1964). A short cold-storage period is beneficial for seedling growth and development. Since *C. tuberosus* has a wide distribution, northern plants may need a longer cold-storage requirement than plants from Florida.

Acclimatization

Six-month old seedlings of *C. tuberosus* were easy to acclimatize under greenhouse conditions. In culture most terrestrial orchids typically germinate and grow slowly (Stoutamire, 1964). Whitlow (1996) did not acclimatize *C. tuberosus* seedlings until they were 15-months old. The findings of this study are in accordance with Myers and Ascher (1982), who acclimatized seedlings under greenhouse conditions after one or more leaves fully expanded. In this study, original shoots died soon after greenhouse culture, but corms persisted. Interestingly, new shoots were formed within several weeks of shoot senescence. Since *C. tuberosus* has a perennial growth habit with one new growth starting in subsequent years, production of new shoots within several weeks was surprising. This suggests that corms were not dormant after leaf senescence.

No significant differences among seedlings acclimatized in potting media were found for leaf and root length as well as leaf production. Root production was significantly lower on seedlings grown in sphagnum than the other potting media. The lower production of roots; however, did not adversely affect seedling growth and development, suggesting that root number may not be important for overall growth and development of *C. tuberosus*. Low root production in sphagnum may have been influenced by the production of longer roots. Nutrients may have been allocated for

elongation instead of root production. Both fresh and dry weight and corm diameter were significantly lower on seedlings grown in vermiculite. Decreased weights and corm diameter on seedlings in vermiculite may have been influenced by the quick draining medium. The data from this study show that young seedlings of *C. tuberosus* can be grown in any water-retentive yet well-drained growing substrate.

Corm Formation

Calopogon tuberosus grows from a corm and produces a new corm each growth cycle (Luer, 1972). The new corm then produces a new shoot from an apical meristem. Since *C. tuberosus* is perennial, the growth cycle occurs once per year with new shoots actively growing between April and July. In culture, small seedlings began corm production within 12 weeks. During the first 8 weeks, the base of seedlings became swollen (Figure 2-7E). Within 7 months, corms less than 1 cm in diameter formed (Figure 2-17). Corm formation *in vitro* occurred regardless of seedling size, medium type, and light treatment (Figure 2-15 and Figure 2-18). This suggests that initial corm formation may be controlled by factors other than photoperiod. Upon acclimatization original growth died, and new growth commenced within several weeks. Several seedlings formed 2 shoots from the original corm (Figure 2-18 and Figure 2-23E). The start of new growth may have been due to time of year since seedlings were sown in August and acclimatized March through May, which is the active growing season for naturally occurring plants. Whitlow (1990) found that dividing corms of mature plants in half between meristems promoted growth on each small corm section.

Production of *in vitro* tubers of several terrestrial orchid species has been previously reported (Barroso et al., 1990; Debeljak et al., 2002). Jasmonic acid and sucrose combinations were effective in inducing *in vitro* tuber formation in *Pterostylis sanguinea*

(Debeljak et al., 2002). Barroso et al. (1990) found that production of minitubers on several *Ophrys* species was significantly influenced by culture medium type. In this study corms formed on *C. tuberosus* cultured on basic seed germination media. The inclusion of hormones and/or higher sucrose levels may promote formation of larger and more numerous corms (Barroso et al., 1990; Debeljak et al., 2002).

Previous studies have demonstrated that corm tissue of non-orchid species is highly regenerative in liquid culture. Ilan et al. (1995) reported that dormant corm tissue of several *Brodiaea* species regenerated protocorm-like-bodies when cultured in liquid media. Nhut et al. (1994) and Dantu and Bhojwani (1995) reported *in vitro* corm formation of *Gladiolus* using liquid culture. Nhut et al. (1994) also found that shoots cultured on solidified medium formed more numerous corms in a lower temperature (15 C) and light level ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) than a higher temperature (25 C) and light level ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Conclusions and Recommendations

This study demonstrated the benefits of light incubation and culture media for seed germination of *C. tuberosus*. In a commercial setting a seed culture protocol that produces a higher number and larger seedlings in a short period of time is beneficial. To optimize seed germination and seedling development, seeds may be germinated on KC and transferred to P723. Seedlings should be transferred to fresh culture media approximately every 8 weeks to avoid over-growth. Seedlings were also easily cultivated under greenhouse conditions. Given optimal greenhouse conditions of partial shade ($700\text{-}800 \mu\text{mol m}^{-2} \text{s}^{-1}$), high humidity (60-80 % RH), weekly fertilization with 150 ppm N, and frequent watering (4-5 days), young plants should produce new growth in a short time frame.

The reproductive capacity of *C. tuberosus* to form corms *in vitro* provides an opportunity for development of a commercially viable production system. Preliminary results show that *in vitro* derived corms are able to generate new corms and shoots when cut in half and cultured on a medium with an auxin (P. Kauth, unpublished). The unreliable availability of plants is a major impediment to customer acceptance of native orchids. Clonal micropropagation would allow for efficient selection and production of genotypes with commercially valuable characteristics. Corm formation in *C. tuberosus* could provide a model system for automated micropropagation of native orchids using bioreactors. While only seeds from Florida populations were utilized in this study, the reproductive capacity of different genotypes from a wide range of distribution should be studied further. Although asymbiotic seed germination of *C. tuberosus* provides an efficient means to produce large seedlings for commercial purposes, symbiotic seed germination should be explored and implicated for conservation and reintroduction purposes.

CHAPTER 3
SEED CULTURE AND SEEDLING DEVELOPMENT OF *Sacoila lanceolata* var.
lanceolata

Introduction

Most commercially available orchids in the United States are epiphytic (growing on trees). Orchids commonly found at nurseries and discount chain stores include epiphytic hybrids of the genera *Phalaenopsis*, *Dendrobium*, *Oncidium*, and *Vanda* hybrids. Even though approximately 250 orchid species are native to the United States, only a few genera are currently in production. Recent interest in the production of native terrestrial orchids has led to a slow expansion of the native orchid market. Currently, the selection and commercial production of native orchids is concentrated on the genus *Cypripedium*. As the market continues to grow, interest in selection and production of new orchids is expected to increase (Britt, 2000; Griesbach, 2002).

Currently, the major obstacle to the commercial production of native orchids is the rarity and protection of many species. Many of the native orchids in the United States are listed as either special concern, threatened, or endangered by state or federal governments. Collection of seeds and plant material often requires permits from either state or federal governments. Since permits are not often required to collect seed from plants on private property, this is often the seed collection method. High prices for seed-grown plants also hinders customer acceptance of native orchids. In addition, concerns exist that increased awareness together with the high prices of native orchids may encourage illegal collecting.

Approximately 120 species of orchids are native to Florida, comprising almost half the species occurrences in the United States. Many terrestrial species grow along major roadsides. These showy roadside orchids are adapted to variable habitats and disturbances, and are potential candidates for gardens and roadside beautification projects.

Sacoila lanceolata (Aublet) Garay var. *lanceolata* is an attractive terrestrial orchid of subtropical and tropical origins. Plants typically are found in large populations growing along major roads in Florida. Plants form large flowering stems up to 60 cm bearing light to dark red flowers as well as the rarer green and golden color forms (Luer, 1972). A single inflorescence can produce up to 40 tubular shaped flowers. In Florida, the plant flowers in the absence of leaves; however, plants in cultivation flower in the presence of leaves.

An in-depth literature review yielded no published research on seed culture of *S. lanceolata*. Only published studies on the breeding system and taxonomy of *S. lanceolata* exist (Catling, 1987). Catling (1987) noted that *S. lanceolata* is agamospermic, seed formation without pollination by division from ovule tissue, due to a lack of humming bird pollinators in Florida. Agamospermic seed set in each flower is 100%, and agamospermic seed is formed before flowers are fully expanded (P. Kauth, pers. observation). Since agamospermic seed forms from ovule tissue, seeds might possibly produce clones if homozygous. Agamospermic seed culture might provide an efficient method to propagate superior or rare color forms and genotypes. The objectives of this study are to develop a seed culture protocol and outline seedling development of

Sacoila lanceolata var. *lanceolata* by examining the role of photoperiod and culture media.

Materials and Methods

Mature seeds of *S. lanceolata* var. *lanceolata* were collected from Goethe State Forest, Levy County, Florida and Dunnellon, Levy County, Florida in June 2004. Seeds removed from capsules were placed in scintillation vials and stored at 21-23 C in a desiccator with anhydrous calcium sulfate to absorb excess moisture. Seeds were surface-sterilized for 2 minutes in sterile scintillation vials containing 8 ml of an aqueous solution consisting of 0.33% sodium hypochlorite, 5% ethanol (100%), and 90 ml sterile distilled deionized water. Following surface-sterilization, seeds were rinsed three times for 2 minutes each in sterile distilled deionized water. Solutions were drawn out of the scintillation vial with a disposable 1000 µl sterile pipet tip that was replaced after each use.

Seeds were suspended in sterile deionized, distilled water, but the majority of seeds floated. A sterile inoculating loop was used for inoculating culture vessels with the seeds. The inoculating loop was immersed once into the seed suspension and seeds were placed in the culture vessel. The average number of seeds for each individual inoculation was approximately 114 ± 35 .

Media Preparation

Media were prepared two days before seed inoculation. Three basal media (Table 2-2 and Table 2-3) commercially prepared by *Phytotechnology* Laboratories, LLC (Shawnee Mission, KS) were assessed for germination efficiency: Malmgren modified terrestrial orchid (MM) (from Malmgren, 1996), modified Knudson C (KC) (from Knudson, 1946), and *Phytotechnology* orchid seed sowing medium (P723). Malmgren

without pineapple powder was special ordered from *Phytotechnology* Laboratories. Malmgren and KC were standardized to P723 by the addition of 2% sucrose and 0.8% TC[®] agar (*Phytotechnology*, Shawnee Mission, KS) as well as 0.1% charcoal to KC. Media were adjusted to pH 5.7 with 0.1 N KOH before the addition of agar and sterilization for 40 minutes at 1.2 kg cm⁻² and 121 C.

Sterile media were dispensed as 50 ml aliquots into square 100 x 15 mm petri dishes Falcon “Integrid” Petri Dishes (Becton Dickinson, Woburn, MA). The bottom of each petri dish was divided into 36, 13 x 13 mm cells. Of the 36 cells, the interior 16 were used for seed inoculation to avoid media desiccation at the edges of petri dishes. Five of the 16 interior cells were randomly selected for seed inoculation using a computerized random number generator. Ten replicate petri dishes were inoculated per treatment. Petri dishes were sealed with a single layer of Nescofilm (Karlan Research Products, Santa Rosa, CA) to avoid desiccation.

Light Treatments

Effects of five light treatments on seed germination and seedling development were evaluated: 1) 8 weeks under a 16 hour light/8 hour dark photoperiod; 2) 8 weeks continuous darkness; 3) 2 weeks continuous darkness followed by 6 weeks under a 16 hour light/8 hour dark photoperiod; 4) 4 weeks continuous darkness followed by 4 weeks under a 16 hour light/8 hour dark photoperiod; 5) 6 weeks continuous darkness followed by 2 weeks under a 16 hour light/8 hour dark photoperiod. Light was provided by GE F96T12 fluorescent lights at an average of 91.6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ measured at culture level and 25 ± 2 C. Cultures in complete darkness were placed in a light-free cabinet at 24.2 ± 0.6 C. Cultures maintained in 8 weeks light were evaluated weekly for 8 weeks to

determine percent germination. After culture 2 weeks dark, 4 weeks dark, and 6 weeks dark pretreatments cultures were placed under previously described light conditions and evaluated weekly until week 8. Seeds cultured in the 8-week dark period were evaluated once at week 8. Seedling development was recorded weekly using the six stages of seedling development (Table 2-3) as described by Stenberg and Kane (1998). Cultures were viewed under a Nikon SMZ100 Stereoscope (Southern Micro Instruments, Marietta, GA) equipped with a Nikon Coolpix 4500 (Nikon Corporation, Tokyo, Japan).

Statistical Analysis

Germination percentages were calculated by dividing the number of germinated seeds by the total number of seeds in the subsample. The percent protocorms and seedlings in a specific developmental stage was calculated by dividing the number of seeds in that stage by the total number of germinated seeds. Seed germination and seedling development were analyzed using general linear model procedures, least square means, and LSD at $\alpha=0.05$ in SAS v 8.02. Germination counts were arcsine transformed to normalize the variation.

Results

Embryos did not stain in a tetrazolium test suggesting that embryos were not viable (S. Stewart, unpublished data). Seeds slowly became swollen on all media. A low percentage of seeds germinated by week 2 on MM, while seeds began to germinate by week 3 on KC and P723 (Figure 3-2). Germination rates increased weekly during 8 weeks culture on all media. Seed germination was low on all media and in all light treatments (Figure 3-1 and Figure 3-2). No germination occurred in the 2 weeks dark, 4 weeks dark, and 6 weeks dark pretreatments. At week 8, the highest germination occurred on MM incubated in 8 weeks light. The lowest germination occurred on P723

in 8 weeks light. A small number of embryos developed to Stage 2 within the 8-week culture period on KC and MM. Less than 2 percent of seeds developed to Stage 2 on all media and light treatments. During the 8-week culture period embryos and protocorms did not turn green, but rather remained a cream to white color (Figure 3-3). The visual contamination rate was approximately 11%.

After 12 weeks culture seeds began to turn brown and die on all media. All embryos cultured on KC and incubated in both the 8 weeks light and 8 weeks dark died after week 12. After 5 months culture, several embryos developed beyond Stage 1 on MM. Five protocorms developed to both Stages 2 and 3, while one seedling developed to stage 5 (Figure 3-3). Protocorms in Stages 2 and 3 developed abnormally by turning a brown/red color or becoming elongated and hyperhydric (Figure 3-3).

Discussion

Many researchers have reported difficulties germinating seeds of terrestrial orchids (Arditti, et al. 1981; Rasmussen, 1992; Zettler et al., 2001). Terrestrial orchids also might be more dependent on a mycorrhizal fungus than epiphytic species (Stoutamire, 1974), making *in vitro* asymbiotic seed germination difficult. As found in other species, seed germination of *S. lanceolata* was difficult.

The initial germination rates after 8 weeks culture are misleading for *S. lanceolata* since embryos became brown and died after 12 weeks culture. Stenberg and Kane (1998) noted that nearly all seedlings of the epiphytic orchid *Encyclia boothiana* died after 16 weeks when cultured on Vacin and Went medium and Lindemann's orchid medium. This suggests that culture medium type significantly affects the survival of protocorms and seedlings. Due to high seed mortality on all media, embryo death may not have been affected solely by media type.

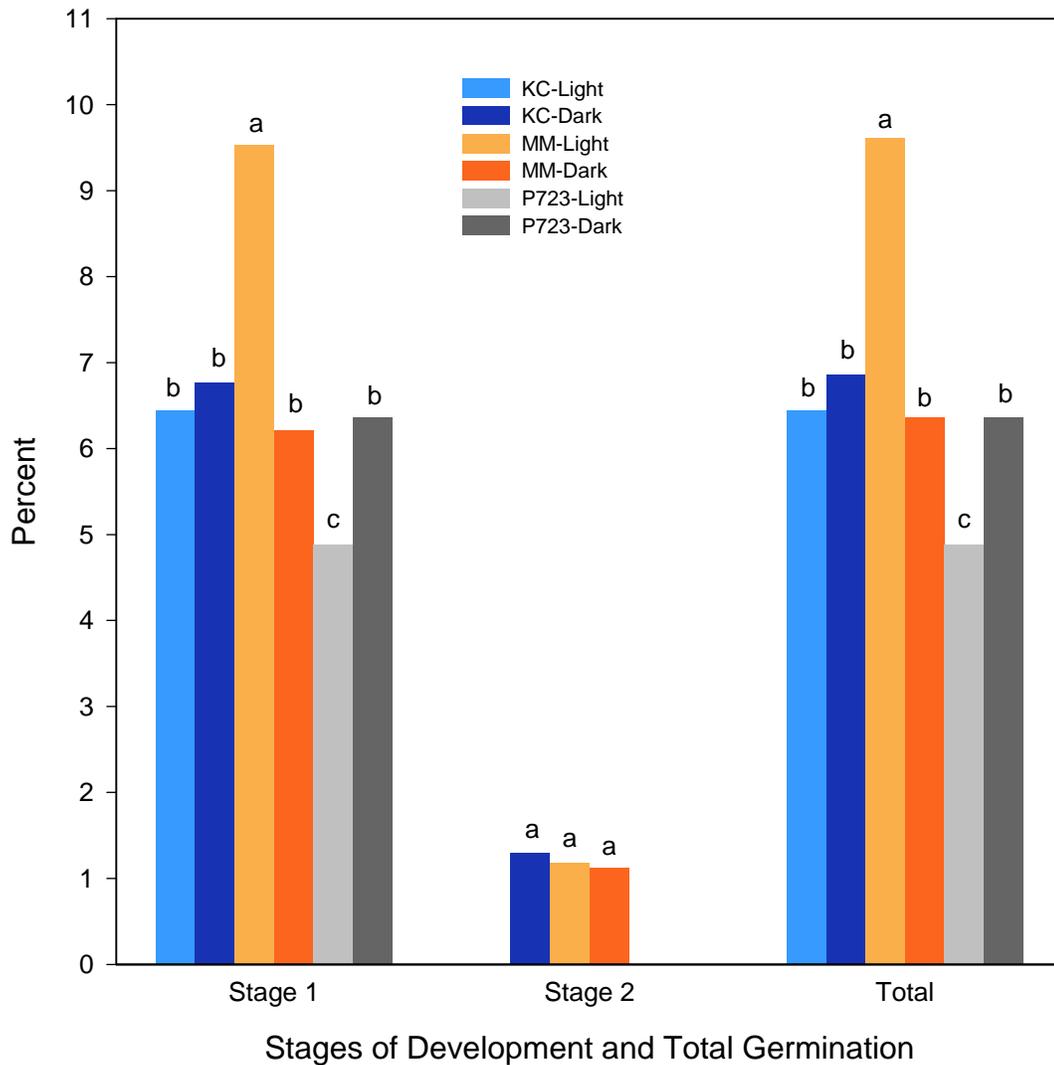


Figure 3-1. Effects of media type and light treatment on seed germination and seedling development of *Sacoila lanceolata* var. *lanceolata*. Seeds were incubated for 8 weeks in either complete darkness (Dark) or a 16-hour light/8-hour dark photoperiod (Light). Each histogram represents the mean response of 10 replicate plates with 5 subsamples each. Histograms with the same letter are not significantly different ($\alpha=0.05$) within each developmental stage. KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium.

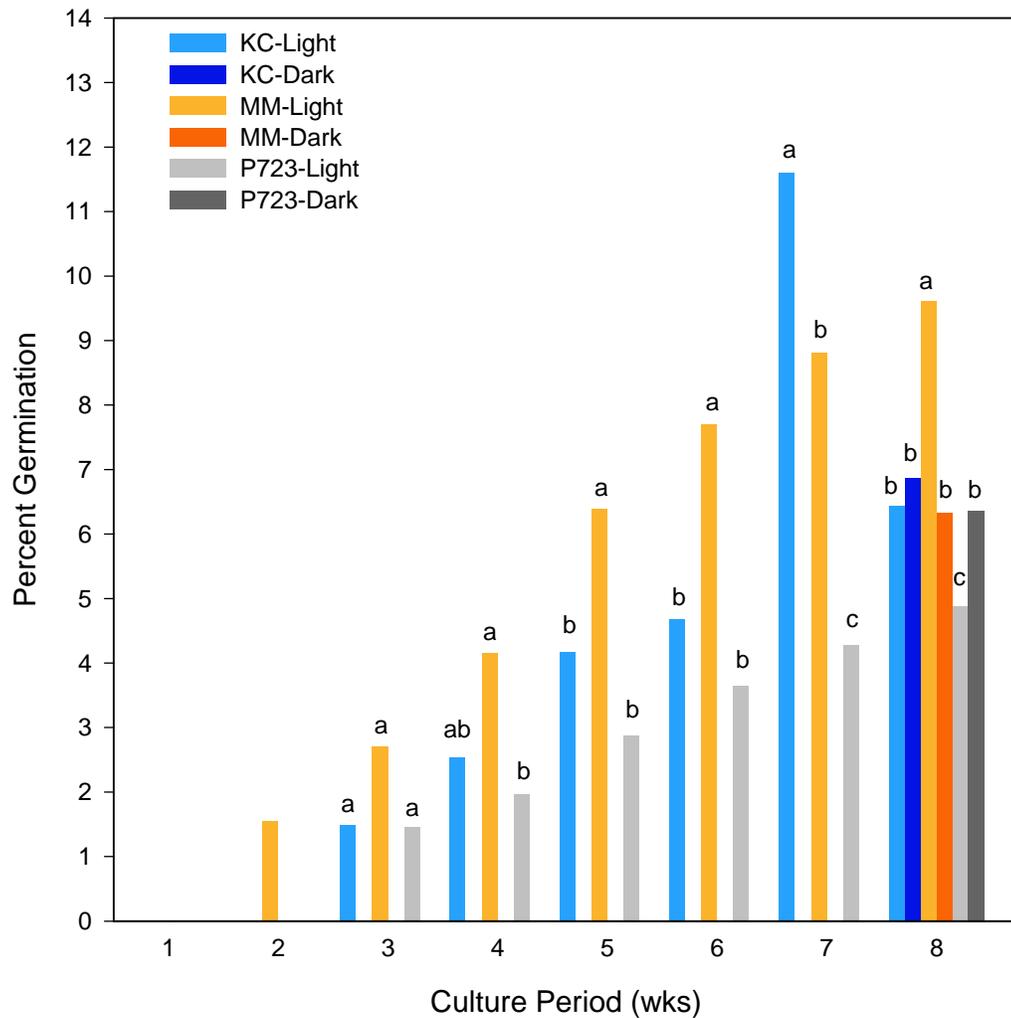


Figure 3-2. Effects of media type and light treatment on weekly seed germination of *Sacoila lanceolata* var. *lanceolata*. Each histogram represents the mean response of 10 replicate plates with 5 subsamples each. Histograms with the same letter are not significantly different at $\alpha=0.05$ within each developmental stage. KC: Knudson C; MM: Malmgren modified terrestrial orchid medium; P723: orchid seed sowing medium.

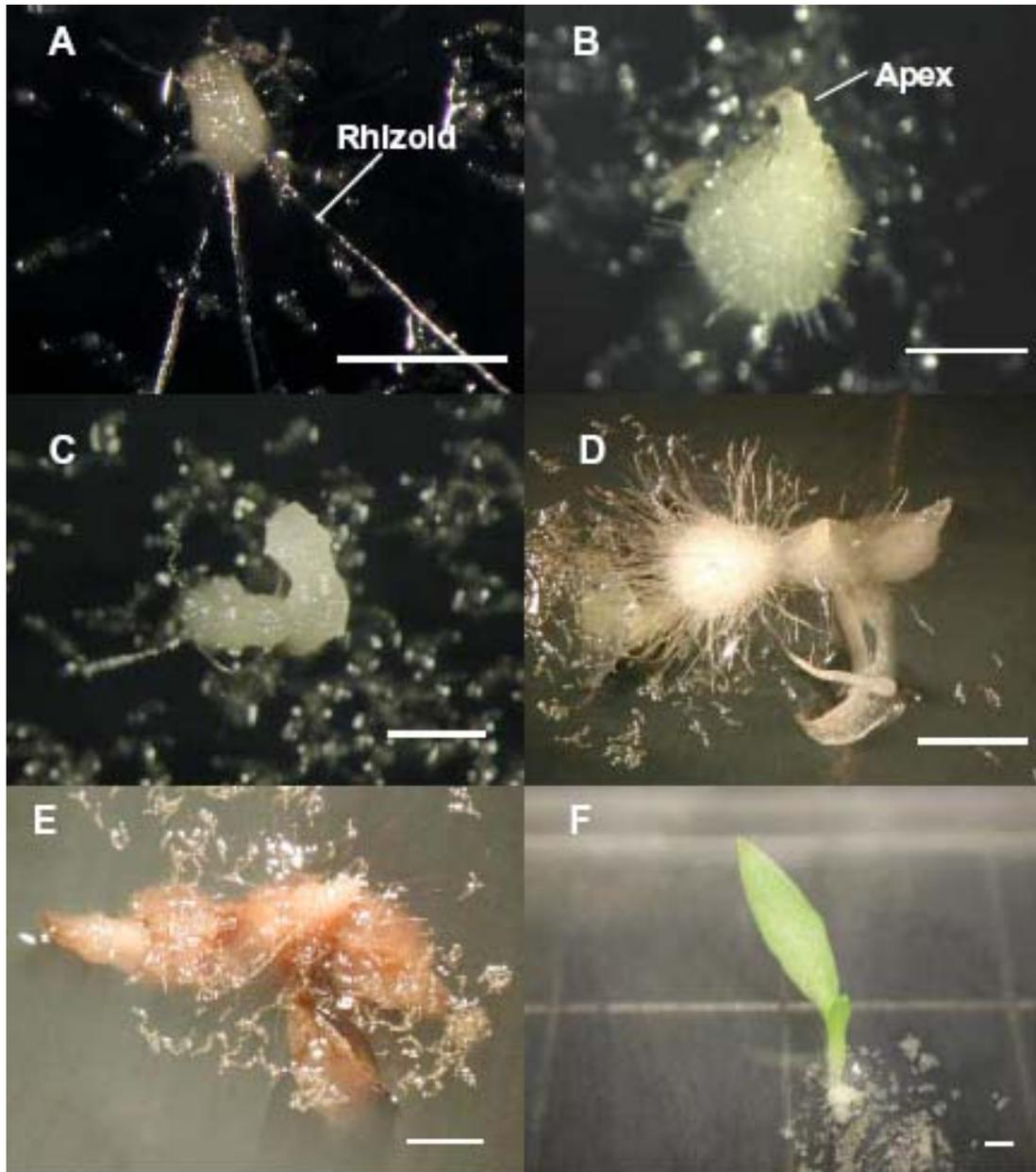


Figure 3-3. Seed germination and seedling development of *Sacoila lanceolata* var. *lanceolata*. A) Stage 2 protocorm with rhizoids. B) Stage 3 protocorm with vitrified shoot apex. C) Elongated stage 2 protocorms. D) Abnormally developed stage 2 and 3 protocorms. E) Abnormally brown/red colored protocorms. Abnormal color often indicates cell death. Note the ungerminated seed surrounding the protocorms. F) Stage 5 seedling with two green leaves. Scale bars=1 mm.

Researchers have found that seed germination of many cold-hardy terrestrial orchids increased at various post-pollination seed collection times. De Pauw and Remphrey (1993) reported that seeds of *Cypripedium* species collected 8 weeks after pollination germinated poorly. They noted that after 8 weeks pollination seeds became light brown and dry. Seeds collected before 8 weeks pollination were white and moist. Van Waes and Debergh (1986b) noted that seed coat permeability, affected by suberin build-up, might influence seed germination. Seeds of *S. lanceolata* also turned light to dark brown, which may be due to suberin accumulation in the testa. Treatment in a hypochlorite solution not only surface sterilizes seed, but also breaks down suberin (Van Waes and Debergh, 1986a, 1986b). Prolonged treatment in either calcium or sodium hypochlorite increased seed germination of *Cypripedium macranthos* (Miyoshi and Mii, 1998) and several Western European orchids (Van Waes and Debergh, 1986b). Longer pretreatment in low concentrations of hypochlorite may cause a further break down of suberin making the testa more permeable (Van Waes and Debergh, 1986b).

Pretreatment with plant growth regulators is often beneficial for increasing rates of seed germination. Harvais (1982) suggested that mycorrhizal fungus might be a source of cytokinins for germinating seeds. Van Waes and Debergh (1986b) found that a cytokinin was essential for the germination of *Cypripedium calceolus* and *Epipactis helleborine*, but not necessary for germination of *Dactylorhiza maculata* and *Listera ovalis*. Seed germination of *Cypripedium candidum* increased with the addition of cytokinins to the culture medium. Miyoshi and Mii (1995) also reported enhanced seed germination of *Calanthe discolor* with pretreatment in various levels of benzyladenine (BA), a common cytokinin used in plant tissue culture. Dimalla and van Staden (1977)

reported that cytokinins assist in lipid mobilization in seeds with high levels of lipid reserves. Since lipids are the primary storage reserves in orchid seed (Arditti, 1967a), cytokinins may play an important role in lipid metabolism of orchid seeds (De Pauw et al., 1995). Germination ceases if lipids are not utilized (Manning and van Staden, 1987).

Abscisic acid (ABA), a compound known to contribute to seed dormancy, could also influence the poor germination of *Sacoila lanceolata*. Van der Kinderen (1987) detected ABA in seeds of two European terrestrial orchids. Abscisic acid may not necessarily lead to dormancy, but the distribution in the seed and cell sensitivity to ABA may affect germination. Also, seed maturity may cause changes in ABA levels. The concentration of ABA was higher in mature seeds than immature seeds of *Dactylorhiza maculata*. ABA may be leached by a pretreatment in a hypochlorite solution.

In nature orchid seeds germinate only upon infection by a mycorrhizal fungus (Zettler and McInnis, 1994). Mycorrhizal fungi might provide germinating seeds with a source of water (Yoder et al., 2000), carbohydrates (Smith, 1967), vitamins (Hijner and Arditti, 1973), and cytokinins (Harvais, 1982). Seed culture with a symbiotic mycorrhizal fungus often increases rates of germination. Zettler and McInnis (1994) reported that symbiotic seed germination in a period of 7 days 16-hr photoperiod followed by 70 days complete darkness of *Platanthera integrilabia* was significantly higher (44%) than asymbiotic germination (7.5%). Rasmussen (1992) reported that symbiotic seed germination rates of *Epipactis palustris* were significantly higher than asymbiotic rates. Attempts to isolate fungus from *S. lanceolata* roots have been unsuccessful (S. Stewart, pers. comm.); however, seeds may germinate symbiotically with a mycorrhizal fungus isolated from other species.

Conclusions and Future Implications

In vitro seed germination of *Sacoila lanceolata* is both interesting and perplexing. The difficulties with seed germination should not hinder the possible introduction of the plant to industry. Pre-treating seeds with hormones, pre-chilling, or longer hypochlorite soaks may improve seed germination. Symbiotic seed germination with a mycorrhizal fungus may also increase seed germination and long-term survival of seedlings. Comparisons between seed germination of mature and immature seed as well as various genotypes should also be studied. Also, analyzing DNA of seedlings might determine whether seedlings are homozygous or true clones. Developed seedlings may provide a source of tissue explants, such as roots and leaves, for established explants of clonal micropropagation of commercially valuable genotypes.

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BIOGRAPHICAL SKETCH

Philip Kauth grew up in Manitowoc, Wisconsin. After high school, Philip attended the University of Wisconsin-Stevens Point (UWSP). During his years at UWSP, Philip developed an interest in orchids, especially the native orchids of the United States. Philip became involved with the Native Orchid Conservation Committee of the Northeastern Wisconsin Orchid Society. Throughout his involvement with orchid conservation, Philip developed an interest in cultivating native orchids. Philip decided to pursue graduate level education, and more specifically orchid research. Philip graduated from UWSP in May 2003 with a major in biology (emphasis in botany) and minor in chemistry. Philip joined the Environmental Horticulture Department at the University of Florida in August 2003. Upon graduating in August 2005 with a Master of Science in horticultural sciences, Philip will pursue a doctoral degree with the Environmental Horticulture Department at the University of Florida. In his spare time Philip enjoys nature photography, music, writing biographies in the third person, and martial arts.